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OSMOTIC RELATIONS IN MOLLUSCAN
CONTRACTILE TISSUESI. ISOLATED VENTRICLE-STRIP PREPARATIONS FROM LAMELLI-
BRANCHS (*MYTILUS EDULIS* L., *OSTREA EDULIS* L., *ANODONTA*
CYGNEA L.)

BY R. L. C. PILGRIM

*Department of Zoology, University College, London**

(Received 28 June 1952)

The bivalve molluscs include marine, estuarine and fresh-water forms, and they yield good heart and cilia preparations. Although the ecological distribution and powers of osmoregulation of several species have been studied in detail, little is known about the effects of osmotic pressure on the functioning of their tissues. At the suggestion of Dr G. P. Wells, the writer undertook a comparative investigation of the effects of osmotic pressure variations on the hearts and cilia of lamelli-branches. The three species used were chosen because they are readily obtained and kept in London, where the work was done, and they represent a wide ecological range. The experiments on hearts are described in the following pages. Those on cilia will form the subject of a second paper.

A study of the osmotic pressure relations of rhythmic muscles from three polychaete species was published by Wells & Ledingham (1940). They drew attention to the physiological importance of rate of change of salinity, as distinct from its absolute value. To distinguish between these factors they made experiments of two types, the 'constant exposure', in which the osmotic pressure is suddenly changed to a new value at which it is then held constant, and the 'drift', in which it changes gradually at a controllable rate. The distinction between the actions of osmotic pressure *per se* and of osmotic pressure change was further emphasized during the course of the present work.

MYTILUS EDULIS L.

The genus *Mytilus* is very widely distributed while *M. edulis* is found on both sides of the North Atlantic. The species occurs over a wide salinity range from full sea water down to below 15%† (Haas, 1926; Dodgson, 1928; Percival, 1929; White, 1937; Conklin & Krogh, 1938).

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† Throughout this paper the concentrations of the sea water are shown as percentage sea water. Data from other workers have been recalculated accordingly on the basis 100% sea water = *S* (salinity) 34.5‰ = Δ 1.88° C. = sp.gr. 1.027 = 3.19% NaCl.

The suggestion has been made that the individuals collected from the lowest salinities, in the Baltic, constitute a physiologically distinct race (Wells, Ledingham & Gregory, 1940). Experimentally, the animals have been acclimatized to external environments of down to 2% sea water (Dodgson, 1930; Topping & Fuller, 1942) and even to fresh water according to Beudant (1816). However, workers with *M. galloprovincialis* (which is sometimes regarded as a subspecies of *M. edulis*) and *M. californianus* have recorded somewhat less tolerance of dilution (Bouxin, 1931; Ricci, 1939; Fox, 1941). Several authorities have compared the osmotic pressure of the body fluids with that of the surrounding water; Monti (1914) found a slight degree of hypertonicity in sea water, but according to Schlieper (1929), Maloeuf (1937), Conklin & Krogh (1938) and Krogh (1938) there is no active regulation in external concentrations down to 29% (Conklin & Krogh 1938) and 20% (Schlieper, 1929). There may be some degree of osmotic regulation at the very lowest salinities at which the mussels occur and to which they can be acclimatized; nevertheless, the demonstrated isotonicity over a wide range of external salinity suggests a considerable resistance of the tissues to osmotic variations.

The cardiac physiology of this species has been little studied. The rate of beat of the heart *in situ* varies from 10 to over 50 beats per minute, and is slowed by closing of the shell (Carlson, 1906; Field, 1922; Woortman, 1926; Diederichs, 1935). The heart of small specimens can be seen through the transparent shell, and Schlieper (1929) found by this means that transfer from an external concentration of 22% to one of 49% reduced the heart frequency to 62–84% of its previous value. As these results were got with whole animals, they may have been due to factors other than the direct action of salinity on the heart tissue.

Methods

M. edulis was obtained weekly from Plymouth, and kept in cool aerated aquarium sea water. The animal was removed entire from the shell, pinned ventral-side down in a dish of sea water and the roof of the pericardium removed. A thread was tied about each end of the ventricle and the auricles cut away, on one side the cut being extended right along the ventricle giving access to the rectum which was then removed. The ventricle was freed beyond the threads and transferred to the apparatus. The preparation consisted, therefore, of roughly the whole ventricle open along one side so that all parts were easily accessible to the bathing fluids. It was attached to an isotonic lever weighted to a tension of 100–150 mg. for specimens of 8–10 cm. shell length. After an hour or two of irregular activity, a regular rhythm was obtained in about 80% of the animals. Such preparations have been maintained active up to 3 days after mounting without aeration or addition of any nutrient; the medium was replaced by new, well-aerated sea water daily.

The preparations were mounted in the apparatus (usually in pairs) by attaching one thread to a fixed glass rod and the other to the kymograph lever. They were immersed in 250 ml. of medium in a beaker resting on a block of wood; by removing the wood and replacing the beaker with one containing a fresh medium a very rapid

and complete change was effected; the new bathing medium thus retained its composition and quickly reached all parts of the preparation. This will be referred to as the 'Constant Exposure' method following the terminology of Wells & Ledingham (1940). Dilution of sea water was made with aerated $M/400\text{-NaHCO}_3$ to maintain the pH between 7.8 and 8.2.

After settling down in Plymouth aquarium sea water (see Appendix), preparations exhibited a very regular rhythm of simple contractions and relaxations whose amplitude was usually steadily maintained for several hours; some gave an unaccountably fluctuating amplitude, particularly with respect to systolic level. The frequency ranged from 11 to 24 beats/min. between 10 and 16° C., the variation appearing to be due largely to differences between individuals.

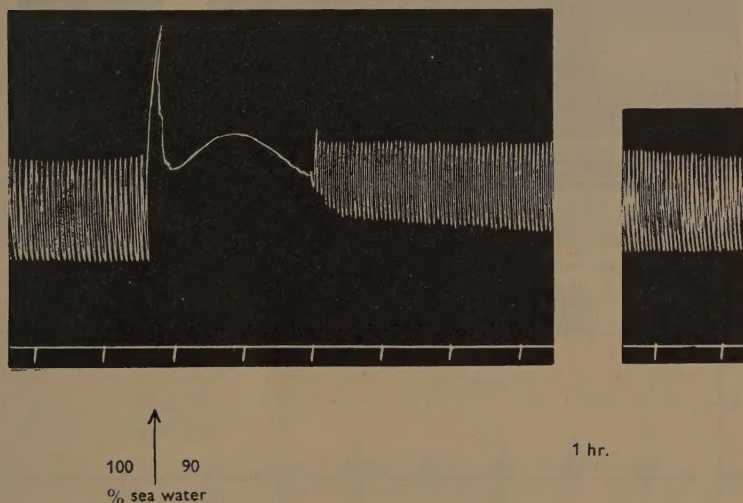


Fig. 1. *Mytilus* ventricle strip preparation transferred from 100 to 90 % sea water. The mechanical effect of change is seen as a spike at the arrow. In all records, read from left to right; contraction is upwards; time is in minutes.

Results

When the beaker of sea water was replaced by another with the same fluid there was a sharp contraction of the preparation, followed by relaxation with gradual resumption of the beat to normal in 1-4 min. This effect was probably mechanical, arising from the extra tension on the tissue when the buoyant fluid medium was suddenly withdrawn; it will be termed the 'mechanical effect of change' (Fig. 1).

The effect of dilution was to produce a contraction following the mechanical effect of change; in cases of moderate dilution it was short-lived and may or may not have been accompanied by inhibition of the beat. This effect was called a 'Shock Reaction' and was followed by accommodation, the beat becoming adjusted in

frequency and amplitude to those characteristic of the new medium. By analysing records at intervals during this accommodation it was found that a steady rhythm developed in 1-3 hr. after the change; this was called a 'Long-term Reaction'. With more dilute media all these effects were correspondingly more pronounced; the

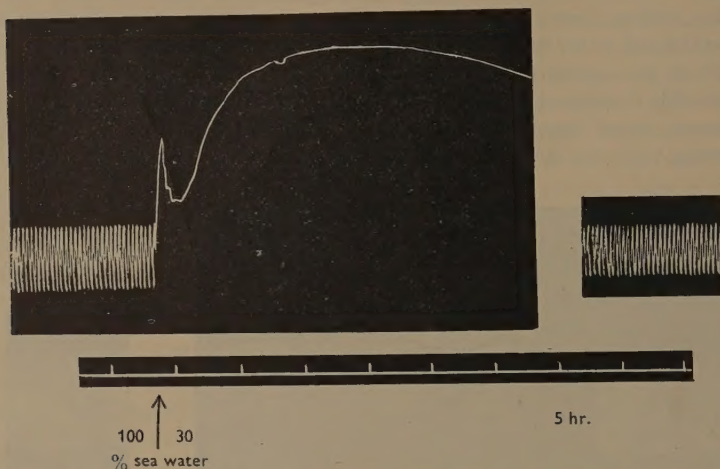


Fig. 2. *Mytilus* preparation transferred from 100 to 30% sea water.

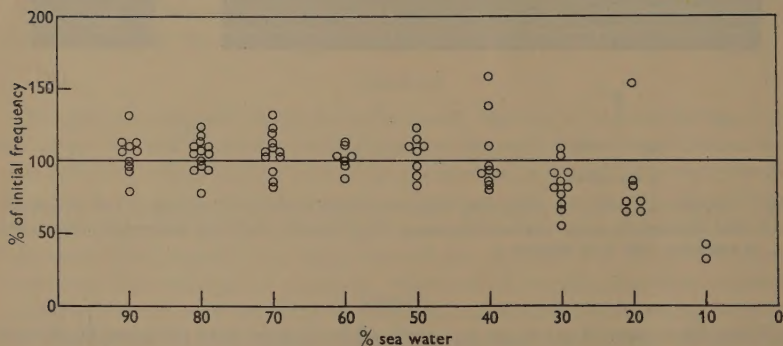


Fig. 3. Analysis of changes in frequency in *Mytilus* preparations subjected to hypotonic sea water; each point represents a separate preparation. Ordinates, final frequency as a percentage of initial; abscissae, concentration of final medium.

contraction and inhibition phases lasted longer and a regular rhythm took up to 6 hr. to appear (Fig. 2).

Fig. 3 shows analyses of frequency for these preparations; the figures have been converted into percentages of those in the initial bathing medium. The lowest concentration of sea water in which even occasional recovery was found was 10%,

though a number of other preparations failed to recover below 30%. In the case of the accommodated rhythm, frequency may be greater or less than that in 100% sea water until 40%, beyond which the trend is towards a decrease. There is no clear correlation of amplitude with salinity.

Those preparations which had accommodated to the diluted media were then replaced in 100% sea water. The shock reaction following mechanical effect of change was, in mild treatments, a rapid return to the normal tone level with inhibition of the rhythm; accommodation to regular beat again took 1-3 hr., the contractions often appearing irregularly at first. When the preparation was returned from more dilute media the shock reaction was more pronounced and included a lowering of tone usually below the normal diastole level (Fig. 4). The increase and decrease of frequency and amplitude were about equally common after this

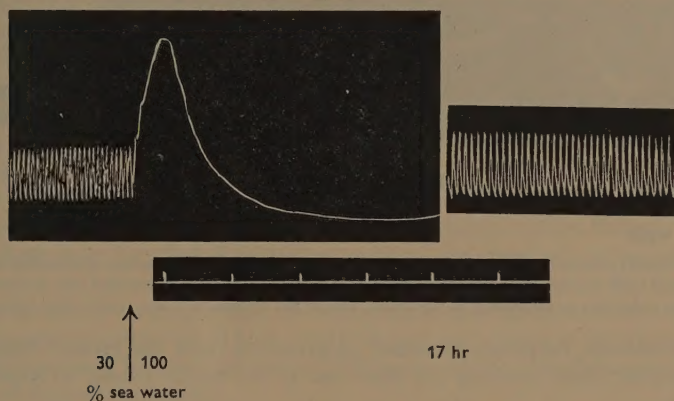


Fig. 4. *Mytilus* preparation transferred from 30 to 100% sea water.
(Same preparation as in Fig. 2.)

treatment; amplitude particularly showed a wide range of response. It was not found possible to accommodate any preparations returned from 10 to 100%. The accommodated beat was commonly less rapid than it had been previously in 100% sea water, especially after great changes in concentration, indicating some degree of damage to the tissue by sudden immersion in very hypotonic media and the return process.

A number of preparations were tested in media consisting of various hypertonic concentrations of artificial sea water (for composition see Appendix). They were first of all transferred from natural to artificial sea water of the same strength; this supported an almost identical rhythm in most cases and these latter were then subjected to hypertonic sea water. The shock reaction following mechanical effect of change was slight for small increases, but for greater increases in concentration there was a contraction with inhibition or reduction of amplitude; this was followed

by gradual relaxation of tone, the beats increasing in size until they became very large and separated by periods of inactivity; finally the preparation remained relaxed (Fig. 5). Accommodation led to a resumption of regular rhythm within 6 hr.; there was accommodation to concentrations up to 160% with no significant change in amplitude and only a slight tendency to reduction in frequency. A number of preparations failed to survive in more hypertonic media and 200% was the limiting concentration; in this, frequency was much reduced in the three successful experiments.

Those which accommodated to the hypertonic media were transferred back to 100% artificial sea water. The shock reaction was in general similar to that described for hypotonic sea water, i.e. a contraction phase predominated, with inhibition of rhythm for periods which were longer for greater changes in concentration. In

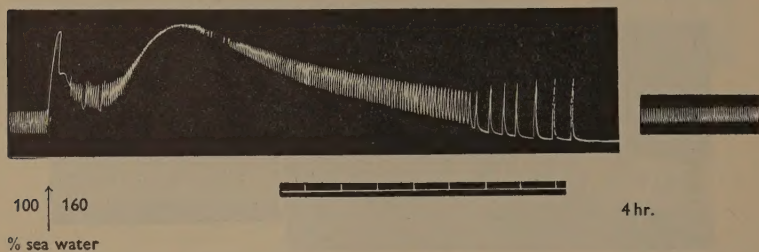


Fig. 5. *Mytilus* preparation transferred from 100 to 160% sea water. After the mechanical effect of change there is a typical example of the Shock Reaction to hypertonic media, viz. a rise in tone (with reduction in amplitude in this case), then a fall in tone to a low level with inhibition.

these treatments frequency increased progressively with the greater change in concentration, thus reversing the effect due to the initial transfer to hypertonic medium.

It appears from these results that the tissue was normally capable of contracting regularly over the range *c.* 40–160% sea water; beyond these concentrations frequency, but not amplitude, tended to lessen and the absolute limits in which any regular activity was present under the experimental conditions were 10–200% sea water.

OSTREA EDULIS L.

The English native oyster, though once an off-shore species, appears now to be confined largely to 'estuarine' beds; nevertheless, it tends to live in positions where the salinity of the water remains fairly high (Russell, 1923; Orton, 1924*b*, 1928; Percival, 1929); further, any individual is unlikely to be subjected to the whole range of salinity variation of an entire area of beds. The species as a whole is commonly exposed to *c.* 70–100% sea water. In estuarine localities there may be considerable temporary dilution as a result of heavy rains, and in some places (e.g. Essex) evaporation over shallow beds during long dry spells may raise the concentration to 107%.

According to Monti (1914) the blood of *O. edulis* is slightly hypertonic in sea water. Krogh (1938) found identity of internal and external chloride concentrations at sea waters of 100 and 72%, and he concluded that the blood came into almost complete ionic equilibrium with the more dilute (Limfjord) water. Oysters can completely close the shell and thus avoid any dilution of the blood for days, even when placed in fresh water (Dakin, 1909); in the case of *O. circumpecta*, which normally lives in 80–100% sea water (Amemiya, 1928), animals placed in 50% sea water resist dilution for some days by this method, but there then follows a gradual dilution of the blood which is ultimately fatal unless the oysters are restored to sea water before a lower limit of about 55% of sea water is reached (Yazaki, 1932). It appears then that this species has no power of osmoregulation, and that the tissues are not at all resistant to dilution of the body fluids.

In ionic composition, the blood of the Japanese *O. circumpecta* closely resembles sea water (Kumano, 1929). The same is true for *O. edulis*; Robertson (1950, personal communication) finds that the most marked deviation is that of potassium, which is 128.7% of that of sea water, but the oyster heart is so tolerant of variations in the ionic ratios (Jullien & Morin, 1931), that this divergence can be disregarded in the present work.

The heart rate of oysters varies greatly, depending on the pH and on the size of the animal among other factors (Pelseneer, 1906; Orton, 1924a; Takatsuki, 1927; Walzl, 1937; Otis, 1942). The effect of varying the total concentration of the medium on the heart beat was studied by Jullien & Morin (1931) and by Hamada (1938), but in both cases the effect of the diluted medium was observed very soon after its application to the heart, and, as shown below, there is a shock reaction in *Ostrea*, just as in *Mytilus*, during which the behaviour is quite different from that after accommodation; moreover, in both cases a 'closed' preparation of the heart appears to have been used, in which the bathing fluids were denied immediate access to the inner surfaces of the heart.

Methods

O. edulis was obtained weekly from Whitstable and kept in cool aerated aquarium sea water. The right valve of the shell was removed (it was advantageous to open the shell via the hinge and cut the adductor muscle away from the dorsal direction), the pericardium slit open and a thread tied about both auriculo-ventricular junctions; a second thread was tied about the ventricle as close as possible to the origins of the aortae, or about the aortae themselves if possible. On cutting the auricles and aortae the freed ventricle contracted and the walls bulged outwards—advantage was taken of this to remove parts of these walls so that the preparation consisted of a double longitudinal strip of ventricle (dorsal and ventral walls), both inner and outer surfaces of which were immediately accessible to the bathing medium. Preparations were mounted in sea water with a lever tension of *c.* 100 mg.; they settled down in a few hours but for convenience were mostly left overnight (*c.* 16 hr.). There was no noticeable difference whether the medium was aerated or not during the experiment, but bubbling was maintained even in the constant exposures because it was used as a stirring mechanism in the 'drift' experiments.

(See p. 305.) In sea water, the frequency on becoming regular varied between individuals from 6 to 18 beats/min.; the rhythm was similar to that in *Mytilus* but a little slower.

Results

Both frequency and amplitude were affected by hypotonic sea water; there was a small initial mechanical effect of change followed by a shock reaction similar to those in *Mytilus*. Inhibition of beat during the contraction phase occurred in 70% and more dilute, and lasted longer the greater the salinity change. Accommodation occurred in dilutions down to 50% sea water, while several preparations recovered in 40% and a few in 30%; in no case was there any activity in 20% even during 24 hr. exposure. The frequency characteristic of the new medium developed in 1-3 hr. and covered a rather wide range at each level of dilution, but there was a distinct trend in the values; dilutions to 80% produced a slight increase in frequency which then fell, until in 40 and 30% the rhythm was considerably slower. This can perhaps be justifiably extrapolated to zero between 30 and 20%. Amplitude distinctly increased with dilution though there was a wide spread in the actual values at greater dilutions.

The return of preparations from 90 and 80% to sea water was uneventful, but from greater dilutions the shock reaction was prominent; it comprised a contraction phase, with inhibition as a rule, then relaxation with a renewed beat vigorous at first but slowing to give irregular isolated contractions and finally relaxation at a low tone level; this picture closely resembled the reaction of the *Mytilus* preparation to hypertonic media (Fig. 5). On accommodation the beat underwent changes clearly reversing its reaction after dilution, i.e. frequency was little altered (average value) until the step 70-100% was reached when concentrating the medium led to an increase.

The preparations beat as well in artificial as in natural sea water, and from the former they were transferred to hypertonic concentrations up to 200%. The shock reaction was slight, without loss of rhythm in the case of 110 and 120%, these being the only concentrations in which accommodation occurred; in 130% and stronger sea water the reaction followed the pattern described for increasing the concentration above (and for *Mytilus*), but there was no accommodation in these cases, though they were quite capable of resuming beat when returned to 100% later. In 110 and 120%, accommodation led to a small decrease of frequency and increase of amplitude; reversing the process led to the opposite effects.

On the whole, then, the reactions of the oyster heart to salinity change resemble those of the blue mussel heart, but, as anticipated in reviewing the ecological data, the oyster heart is the more sensitive, and beats over the more restricted range.

Nature of the shock reaction

A comparison of the shock reactions following the above treatments was brought out by analysing the kymograph records with reference to whether the heart was beating or, if inactive, whether it was relaxed or contracted. The change from 100 to 90 or 80% sea water entailed no loss of rhythm, but beyond here a period of

contracture was usually present and its duration increased with the degree of hypotonicity; it is noteworthy that in a few cases of dilutions to 70, 60 and 50% complete contracture did not occur—these were only *apparent* exceptions because marked tone rises were present at the corresponding times, but rhythmic activity was not completely suppressed. The change from hypotonic to 100% sea water did not inhibit the beat until the 60–100% step; in this and lower groups relaxation became very pronounced and was preceded by a short contracture phase (or a tone rise only, in apparent exceptions).

It was found that similar cycles of events constituted the shock reaction in the hypertonicity experiments, and that it was the direction of the change in concentration that governs this reaction. The shock reaction, then, is a well-patterned set of events related to the sudden change in osmotic pressure of the bathing medium or, almost certainly, to the change in ion-concentration-gradient between tissue and medium. It does not seem to be related to the accommodated rhythm, e.g. in sea water hypertonic beyond 120% the shock reaction was typical but no accommodation occurred.

Effect of gradually changing the medium ('drift' method)

This technique, elaborated by Wells & Ledingham (1940), was used for two reasons: first, to eliminate the mechanical effect of change and, more especially with low concentrations, to eliminate the shock reaction effects occurring with the constant exposure technique; the curve of rate of change of concentration lies, even in the rapid drifts, in the region well beyond that of any shock reactions of the constant exposure method so that the preparations may be considered as having accommodated to the drift as it proceeded; secondly, such an approach might extend the range of dilution in which the isolated heart would function. The procedure was to allow the preparation to settle down in 100% sea water, then to run in $M/400\text{-NaHCO}_3$ at a steady rate from a Mariotte bottle (for details see Wells & Ledingham). The frequency and amplitude were measured at approximately hourly intervals and plotted on a graph against the concentration of the medium as calculated from calibration curves for the apparatus. At the end of each experiment the final concentration was checked by titrating total chlorides with silver nitrate; the results were rejected unless the analysed and calculated concentration values agreed within 4% sea water.

The results of two groups of experiments are shown in Figs. 6 and 7; in most the rate of change was such that the concentration reached 50% in $t_{\frac{1}{2}} = 3\text{--}3\frac{1}{2}$ hr.; in others $t_{\frac{1}{2}} = 6\text{--}8$ hr. The trend of the curves for frequency is similar in both groups, though the slowly drifted ones had a less-marked tendency to increase initially. The rapidly drifted preparations clearly showed an increase in frequency which was maintained as far as 50% sea water or occasionally 40%; there was a rapid falling-off in rate beyond this level, and many preparations passed their maximum frequency well before this. Comparing these results with those of the constant exposure method the range of individual variation was remarkably similar, indicating that the variation was biological rather than experimental, while the trend of the

curves 'frequency \times concentration' was the same. Very little extension of the lower end of the curve was obtained by either fast or slow drifts; a few preparations were feebly active below 30%, but none reached 20% and these low concentrations

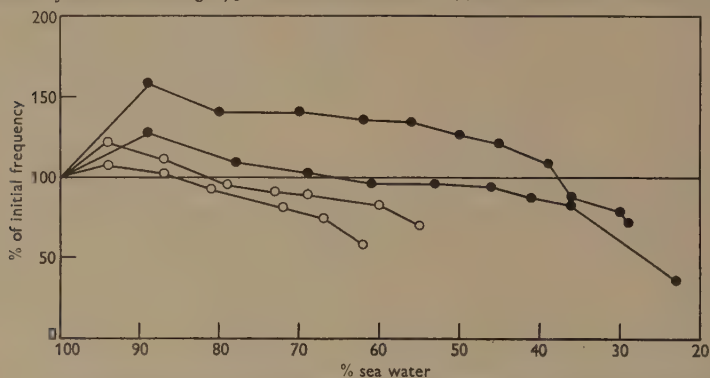


Fig. 6. *Ostrea*; change in beat frequency of ventricle strip preparations subjected to hypotonic sea water by the Drift method. In Figs. 6 and 7, solid circles represent rapid drifts; open circles, slow drifts. In each group the examples shown cover the range of variation found.

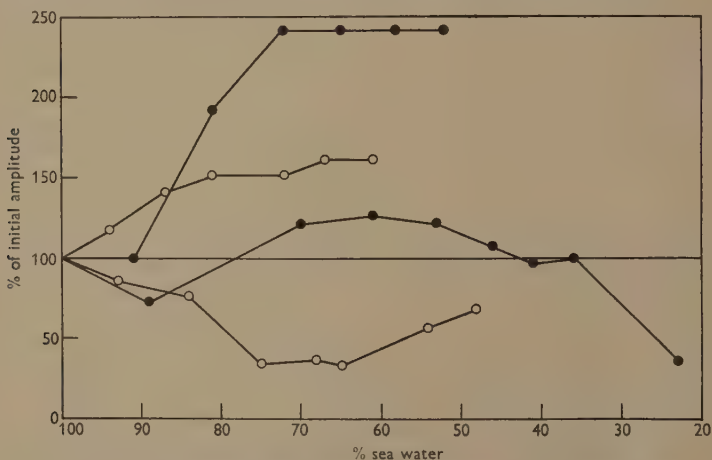


Fig. 7. *Ostrea*; change in beat amplitude of preparations undergoing Drift. See Fig. 6 for explanation.

must be considered well beyond the range of this tissue. The oyster heart resembles the rhythmic muscles of polychaetes studied by Wells & Ledingham (1940), in that the same lower salinity limit is given by the 'drift' and 'constant exposure' methods. The curves 'amplitude \times concentration' (Fig. 7) were very scattered; fast and slow drifts were not segregated in this respect, and the scatter as a whole resembled that obtained with the constant exposure method.

ANODONTA CYGNEA L.

Fresh-water mussels of the group Unionidae occur in fresh waters throughout the world, the chief variations in their chemical environment being the calcium and magnesium concentrations which may be quite high in regions of hard water. Evidence of their penetration into brackish water is scarce; Haas (1926) records *A. cygnea* from 8% sea water in Randersfjord (east Denmark), and from 14% near Stockholm, while Välikangas (1933) found some at Helsingfors in 13% but stated that they were '...abgestorbene'. Philippon, Hannevert & Thieren (1910) acclimatized *A. cygnea* to fresh water to which had been added sea-salts raising the concentration to nearly 60% sea water.

The osmotic pressure of the blood has been measured by numerous workers and is known to be exceptionally low ('...der niedrigste bis jetzt bekannte Wert', Koch, 1917). Picken (1937) gives a series of sixteen individual determinations; expressed as % NaCl solution isotonic with the blood they range from 0.05 to 0.14;* these values correspond with c. 1.5–4.0% sea water. His general conclusion is that 'the blood has a vapour pressure equivalent to that of a solution of c. 0.010% NaCl'—i.e. c. 3% sea water—and his values are amply confirmed by those of other workers who have studied the blood of the species (Dakin, 1912; Călugăreanu, 1915; Koch, 1917; Damboviceanu, 1924; Duval, 1925; Jatzenko, 1928; Florkin, 1935). Although the osmotic pressure of the blood is low it is considerably greater than that of the surrounding fresh-water medium, so that in its normal habitats the animal actively maintains $\Delta_i > \Delta_e$, i.e. is then homoiosmotic. When the concentration of the external medium is increased, however, the animal soon fails to maintain this hypertonicity and the blood comes to have the same osmotic pressure as that of the outer medium (Călugăreanu, 1915; Duval, 1925; Florkin, 1935; Duchâteau & Florkin, 1950). The animal in these conditions is, therefore, poikilosmotic and its tissues, both internal as well as external, are being bathed by media of increased concentration.

Several workers have studied the heart beat of *A. cygnea* and of other species, either in the intact animal or in media varying from tap water to frog Ringer diluted to 25%, but there has been no study of the effects of varying the salinity of the bathing medium. The heart beat is always very slow, ranging at room temperatures from about 3 to 10 beats/min., and is much slower when the shell is closed than when it is open (Willem & Minne, 1898; Keber, 1851; Noyons, 1908; Koch, 1917; ten Cate, 1923*a, b*; Berthe & Petitfrère, 1934; Hers, 1943; Hendrickx, 1945).

Methods

A. cygnea were used throughout the investigation; they were obtained from Surrey and kept in running tap water for no longer than 1 month before using. Florkin, Duchâteau & Leclercq (1950) have shown that there is no significant change in the blood ionic composition on starving *Anodonta* for this length of time.

* The figure 1.14 in his table 1 is a misprint for 0.14 (Picken, personal communication).

The animal was removed entire from the shell, the pericardium opened dorsally and the ventricle slit from end to end along the mid-dorsal line; the rectum was removed where it runs through the ventricle and the latter separated into two halves by cutting along the mid-ventral line. Threads were tied at each end of each half-ventricle, the auricles cut away and the ventricle sectioned beyond the threads. In this way two symmetrical halves of the organ were obtained, a situation very convenient for conducting adequately controlled experiments since the two halves proved to be physiologically identical. Each half, termed a 'ventricle-strip', was mounted in a beaker separate from its counterpart, but usually together with a strip from another mussel. Under the action of the isotonic lever (tension *c.* 200 mg. for mussels of 12–13 cm. shell-length) the preparations relaxed slowly and were allowed to settle down for 24 hr. after removal.

Results

(i) *In diluted sea water*

Sea water diluted to 4% was chosen as a convenient standard solution, having an osmotic pressure within the range of *Anodonta* blood, although its individual ionic concentrations are somewhat different (see Appendix).

This proved to be an excellent bathing fluid for the ventricle strip. It supported for several days a regular rhythm comparable to that found *in vivo* by other workers, even without any added nutrient or aeration. During the period allowed for the strip to settle down, it relaxed considerably and showed at first very irregular beats which were often of large amplitude, and the mechanical activity was interspersed with relaxation periods of varying length. This may be compared with the results of Motley (1934) on American fresh-water mussels where the heart, exposed *in situ*, passed through four phases, viz.:

- (a) Beats regular and even; lasted only a few minutes after opening shell.
- (b) Beats irregular and erratic; lasted 2–24 hr.
- (c) Beats regular and uniform; lasted 2–93 hr.
- (d) Beats becoming smaller, slower and finally irregular with the onset of fatigue.

In the present experiments phase (a) was not found, no doubt due to the much more rigorous treatment necessitated by complete isolation of ventricle strips—the other three phases correspond well with those found in the present investigation, though regular uniform beats have been obtained continuously for up to 8 days after removal of the strip, i.e. 7 days after settling down. This is the longest survival and was obtained with four preparations; 4–5 days was the average survival time. Hendrickx (1945) also found periods of irregularity both before and after the regular rhythm was established in *A. cygnea*. The rhythm on becoming regular in 4% sea water was uniform though very slow; of approximately 100 hearts (200 preparations) most fell within the range 1.6–5.5 beats/min. at $T=8-20^{\circ}\text{C.}$, with the following exceptions: 6.4 (13°C.), 8.1 (14°C.), 9.1 (14°C.), 8.3, 9.0, 12.6, 13.0 (all at 21.5°C.).

The mechanical disturbance produced by the constant exposure technique was very slight and often not noticeable in the case of *Anodonta* preparations—this was

probably due to their larger size, as compared with *Mytilus* and *Ostrea* ventricles, so that stretching on removing the buoyant medium was insignificant (Fig. 8).

On increasing the concentration of the medium the preparations showed a loss of tone (Fig. 9), accompanied in the higher concentrations by the absence of any mechanical activity. They remained in this relaxed state for several hours in the cases of great changes then gradually began to beat, irregularly at first, until a regular rhythm was once more produced. The whole process of accommodation

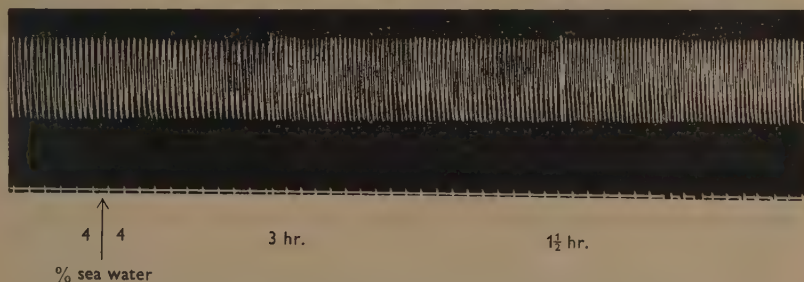


Fig. 8. *Anodonta* ventricle strip preparation transferred from one vessel of 4 % sea water to another; the mechanical effect of change is extremely slight in this animal.

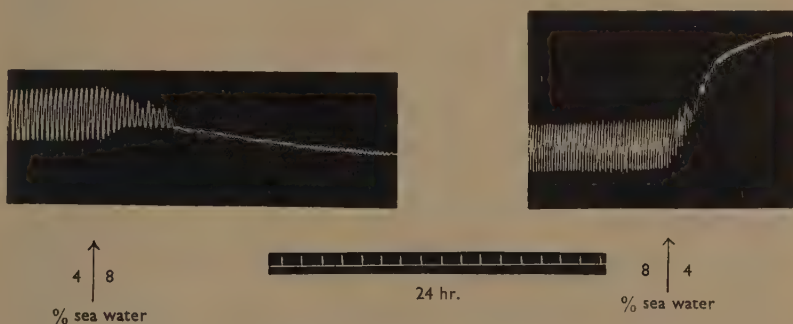


Fig. 9. *Anodonta* preparation transferred from 4 to 8 % sea water and back.

was slow, but short recordings taken at intervals showed that it was adequate to leave the preparations for 20–30 hr. after a change of medium. The effect of increasing the concentration of the sea water in simple multiples of 4 % showed very clearly an increase in frequency of the beat (though its extent was not proportional to the concentration) and a decrease in amplitude, with some exceptions in the 4–8 % group. The highest concentration in which preparations recovered was 40 % sea water, representing a tenfold increase in ionic concentration of the medium.

Studies were also made upon the effect of decreasing the concentration of the medium by returning the accommodated preparations to 4 % sea water. The

immediate reaction was a rise of tone, progressively greater as the change was more severe and often enormous after returning from 20% or stronger sea water. During this tone rise rhythmic activity was inhibited in all cases except some returns from 8%. After further accommodation the beat became characterized by a reduced frequency and usually an increased amplitude.

A number of preparations were treated in the reverse manner, the initial bathing medium being replaced by more dilute sea water, viz. 2 or 1%. Dilution led to a decrease in frequency, while the return to 4% was accompanied by an increase; this is clearly in line with the results of the above experiments and illustrates the relationship between frequency and ionic concentration of the medium. The data for the effect on amplitude do not show the same correspondence; it was more common for dilution to 2% to produce a smaller than a larger beat, while in the case of 1% the beat size became irregular. (A number of other preparations became quite inactive in 1% sea water and resumed beating only after returning to 4%.) It is not considered that these latter results are seriously at variance with the general conclusion that frequency is directly, and amplitude inversely, related to the medium concentration; if it is agreed that 4% sea water is a reasonable blood substitute, at least in respect of its concentration, then 2% and particularly 1% are too dilute in one or more ions to support *larger contractions*. It is always possible, however, to reduce the *frequency* and if this is dependent on ion concentration(s) it will occur with dilution of the medium.

Tap water failed to support heart beat in the preparations used in this work; the success reported by ten Cate (1923*b*) may be related to his use of a different type of isolated preparation or to differences in tap waters.

As in *Ostrea*, preparations submitted to the drift treatment confirmed the results of the constant exposure method, in respect to the dependence of frequency and amplitude on concentration.

(ii) *In diluted Frog Ringer* (see Appendix).

Several workers have used this solution, considerably diluted, as a bathing medium for *Anodonta* hearts. Experiments were therefore carried out to compare the effect of this medium with that of 4% sea water. Sudden transfer from 4% sea water to 1/15 Frog Ringer (R/15) led to an immediate rise of tone with inhibition of beat—there was no sign of mechanical activity within 24 hr. Similar treatment with undiluted Ringer caused inhibition with relaxation, but in 24 hr. there was in some cases a rapid but minute and irregular beat. Now Frog Ringer has a $\Delta = 0.45^\circ \text{C}$. (Clark, 1927, p. 124), so that it is obvious from earlier experiments in this section that R and R/15 are, respectively, too concentrated and too dilute to be suitable for this preparation; indeed their shock reactions show this also. Further experiments were then performed with R/5, which has a $\Delta = 0.09^\circ \text{C}$. (calc.) approximating to those of *Anodonta* blood and 4% sea water; in this case activity was maintained, sometimes with periods of irregularity following the change, but settling down to a steady uniform beat within 24 hr. Nevertheless, survival of the preparations was much shorter (seldom longer than 2 days) than in 4% sea water.

The levels of systole and diastole in R/5 often fluctuated even when the rhythm was otherwise regular; this further suggests that R/5 is not as suitably balanced physiologically for the preparation as is 4% sea water. Similar fluctuations in tone were found by ten Cate (1923*b*, figs. 3, 4) who stated that R/4 favoured the production of this effect.

(iii) *In artificial blood mixture* (see Appendix).

A solution was prepared having an inorganic chemical composition within the range of values given by Florkin *et al.* (1950) for the analysis of *Anodonta* blood. On replacing the 4% sea water by this artificial blood there was little or no shock reaction, and the preparations continued beating with no sign of interruption. The rhythms of six different preparations compared before and 21–24 hr. after such a change showed an increased frequency (133–208%, average 177%); while amplitude was in two cases increased (118%), in three others decreased (68–91%) and in one case remained unchanged (average for the six examples 94%).

Thus an artificial solution made to imitate *Anodonta* blood in respect to cation concentrations was eminently satisfactory when used on the ventricle strips, supporting a rhythm considerably faster than did 4% sea water, but of amplitude averaging about the same. It is to be noted that this greater frequency is still within the normal range for *Anodonta*, and also within the range found for isolated preparations in 4% sea water.

DISCUSSION

Sea water proves to be a suitable medium for the hearts of the two marine species, survival with good activity being possible for 2–3 days. An artificial solution containing the four major cations (as chlorides) in about the same proportions as in sea water is also satisfactory, and for these experiments there appeared no reason to attempt closer imitations of the bloods. Both marine species have a small degree of ionic regulation in that potassium is maintained some 20% above sea-water level and a few preparations of both *Mytilus* and *Ostrea* were tested in sea water with this added potassium; there was no significant difference in the rhythms. The excellent results with *Anodonta* in 4% sea water are intriguing in that this solution has more sodium and magnesium, vastly less calcium and about the same potassium as *Anodonta* blood (see Appendix), though the Δ 's are equivalent on calculation. Furthermore, the preparation beats merely a little faster in an artificial *Anodonta* blood but does not survive here so long (perhaps having used up its energy source sooner). The increase in frequency may be a reaction to extra calcium, though it is certainly not proportional to the difference in this ion; or more probably to the lower concentration of magnesium, an ion which is known to have a depressant action elsewhere. In 1/5 Ringer also, the beat is faster and survival time shorter; here there is comparatively little calcium and no magnesium, which supports the idea that the relatively large dose of magnesium in 4% sea water keeps the frequency low. In concentrations of sea water greater than 4% the more rapid beat must be determined by another ion(s) acting antagonistically to magnesium.

Motley (1934) showed that on perfusion of fresh-water mussel hearts only a three-fold increase of calcium was tolerated, but more systematic work is needed to determine the regulation by the ions and their antagonisms.

When the results are considered as a whole, the general relationship emerges that the ventricles beat faster or slower as the surrounding medium is in greater or lesser concentration, with a falling-off of frequency at extremes. Within the ranges of sea water permitting beat in the marine species there are narrower limits in which the frequency is maximal (*Mytilus* 40–160%, *Ostrea* 70–100%), but the frequency of *Anodonta* continues to increase up to the maximum capable of supporting beat (40%).

Amplitude shows some tendency to behave in an inverse manner (*Ostrea* and especially *Anodonta*), but differences between individuals are large and this property is capricious and does not lend itself to strict quantitative analysis.

It is interesting to compare the properties of the heart preparations with other characteristics of the three animals (Table 1). The comparison suggests that there

Table 1. *Comparison of properties of the whole animals and of the heart preparations for the three species*

	<i>Mytilus</i>	<i>Ostrea</i>	<i>Anodonta</i>
Activity of animal	Semi-sessile but shell movements vigorous	Sessile but shell movements vigorous	Non-sessile but very sluggish
Oxygen consumption, ml./kg./hr. (Heilbrunn)	54.9	15	2
Heart beat	Rapid	Less rapid	Very slow
Beats/min.	11–24	6–18	1.6–5.5
Blood concn. approx. (as % sea water)	100	100	4

is a direct correlation between blood concentration and the whole metabolism of the animal; that the dilute internal medium of *Anodonta* calls the tune for its low heart rate and general activity; for example, subjecting the isolated ventricle to concentrated media causes it to develop a rapid beat, while dilution slows it down. With *Ostrea* and *Mytilus* the same relationship holds for dilutions below 100% sea water. It would be interesting to know what the activity of whole *Anodonta* was in experiments where they were acclimatized to dilute sea water.

The results of the experimental work with respect to salinity tolerance of the tissues and the data for ecological ranges from the literature are summarized in Table 2. In *Mytilus* there is no experimental evidence of osmoregulation but this probably occurs in very dilute sea water, so that in all other conditions the heart is bathed from both sides by the same medium (in terms of inorganic ions); it is shown capable of approximately normal activity over a range greater than that found in the ecology of English specimens. There is a particularly striking adaptability of the tissue to hypertonic media, and even much beyond the range supporting normal activity it is not killed by the treatments. These facts suggest that

slow decreases and increases of salinity could probably be withstood experimentally by the species to as far as Fox found with *M. californianus*.

Ostrea edulis does not appear normally to live in sea water below 70%, so that although osmotic regulation does not operate, except perhaps in much more dilute conditions, the heart may never in an intact animal be subjected to a body fluid more dilute than this. It is significant that the frequency of the heart beat in these experiments is maximal in those concentrations which the heart may experience normally, while in more dilute media the beat becomes very slow. Similarly, the amplitude, while not showing such a clear trend, becomes very irregular at low concentrations.

Anodonta is the only species constantly to maintain $\Delta_i > \Delta_e$. Were it not to do so as successfully, it seems that its heart beat would become so extremely slow as to be almost ineffective in circulating the blood. The finding that the beat is not

Table 2. *Salinity tolerance of the tissues used in the experiments, and the ecological ranges of the species*

(All figures are in % sea water)

	Ventricle strip		Ecological range	
	Activity good	Absolute limits	Natural	Experimental
<i>M. edulis</i> (England)	40-160	10-200	45-100	—
<i>M. edulis</i> (Baltic)	—	—	15-?	15-30
<i>M. californianus</i> (U.S.A.)	—	—	—	33-200
<i>O. edulis</i> (England)	70-120	30-120	70-100	—
<i>O. edulis</i> (France)	—	—	—	70-100
<i>Anodonta</i> spp. (Europe)	2-c. 24	1-40	Fresh water-c. 14	Fresh water-c. 60

maintained beyond 40% sea water, whereas Philippon *et al.* (1910) accommodated the animal to c. 60% is reasonably explained by the slow acclimatization possible with whole animals; this is precluded in experiments on isolated tissues, but obviously the heart must have been beating in Philippon's experiment and thus functioning in a very hypertonic medium.

The overall picture is that the tissues studied are capable of functioning well in fluids of both greater and lesser concentration than those normally bathing them, though extremes of concentration support reduced levels of activity. These capabilities may be considered as safety margins, allowing latitude in the case of a temporary breakdown or overshoot in some regulating mechanism.

There is evidence, then, that adult animals of the three species can survive quite wide fluctuations of salinity of their environmental media; restriction of their ecological distribution in respect to salinity, therefore, seems to be related to some other factor(s), e.g. less tolerant stages in the life histories, as suggested by Needham (1930).

SUMMARY

1. An investigation was made into the effects of variations of osmotic pressure of the bathing fluid on isolated ventricle preparations from three species of lamellibranchs.

2. Suitable media were found in sea water for *Mytilus* and *Ostrea* and 4% sea water for *Anodonta*. Under the conditions described, a regular beat resembling the natural beat is maintained for days, although the media used differ somewhat in ionic composition from the natural body fluids.

3. Sudden changes in concentration of the bathing media lead to temporary 'Shock reactions' followed by accommodation. The shock effects can be avoided by gradual changes in concentration, the final effects being essentially the same whether the change is gradual or abrupt.

4. In all species, the shock reaction included reduction in amplitude or even inhibition of the rhythm, and also the following effects, which depend on the direction of change of concentration. Change from a normal to a hypotonic medium, or return from a hypertonic to a normal medium, caused tone rise or systolic contracture; change from a normal to a hypertonic medium, or return from a hypotonic to a normal medium, caused tone lowering or diastolic relaxation.

5. The beat after the shock reactions had passed off was in general faster in hypertonic and slower in hypotonic media. In the marine species, however, there was also slowing in the most concentrated media capable of supporting a beat.

6. A comparison is drawn between features of the activity and metabolism of the three species and the concentration of their bloods. It is suggested that the very dilute blood of the fresh-water mussel restricts its heart rate, general activity and metabolism.

7. The tissues investigated are shown to tolerate ranges in the concentration of media wider than those encountered under natural conditions, and it is suggested that salinity of the environment is not a critical factor in restricting the ecological distribution of adults of the species. The tolerance of the tissues may have a safeguarding effect in the event of temporary inefficiency in the osmoregulatory mechanism.

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APPENDIX

Ionic composition of the body fluids and of the media used. All in grams/litre

	Na	K	Ca	Mg	Cl	SO ₄	Δ (° C.)	pH	Reference
Plymouth aquarium sea water, 100 %	11.27	0.398	0.545	1.38	20.19	2.81	1.97	8.1-8.2	Hayes & Pelluet (1947)
Plymouth aquarium sea water, 4 %	0.506*	0.0159	0.0218	0.055	0.808	0.112	0.079	8.2	—
Artificial sea water, 100 %	11.16	0.42	0.45	1.42	20.07	—	—	8.0	—
Frog Ringer (R)	2.45	0.064	0.045	—	3.92	—	0.45	8.4	—
Frog Ringer (R/5)	0.536*	0.0128	0.009	—	0.784	—	0.09	8.3	—
M/400-NaHCO ₃	—	—	—	—	—	—	—	8.2-8.3	—
Tap water	—	—	—	—	—	—	—	8.1	—
Distilled water	—	—	—	—	—	—	—	5.4	—
Anodonta; blood substitute	0.369	0.010	0.366	0.0089	1.1685	—	—	8.1	—
Anodonta; blood	0.356	0.0155	0.212	0.0086	0.370	0.147	—	—	Hayes & Pelluet (1947)
Anodonta; blood	0.372	0.010	0.365	0.0090	0.427	—	—	—	Florkin <i>et al.</i> (1950)
Anodonta; blood	± 0.014	± 0.0012	± 0.027	± 0.001	± 0.020	—	—	—	Duval (1924)
Anodonta; blood	—	—	—	—	—	—	—	7.8	Jatzenko (1928)
Mytilus edulis; blood	10.34-10.36	0.35	0.460	—	19.2	—	—	8.02-8.08	Bethe & Berger (1931)
Sea water	10.75	0.432	0.420	1.13	18.86	—	—	—	—
Mytilus galloprovincialis; plasma	12.92	0.560	0.522	1.490	22.76	3.87	—	—	Robertson (personal communication)
Sea water	12.79	0.463	0.489	1.544	23.06	3.219	—	—	—
Ostrea edulis; plasma	12.57	0.589	0.486	1.557	22.72	3.165	—	—	—
Sea water	12.64	0.458	0.483	1.525	22.78	3.18	—	—	—
Ostrea sp.; haemolymph	—	—	—	—	—	—	—	0.3-0.4 below external medium	Jullien & Richard (1936)

* Includes Na from the NaHCO₃ used in dilution.

OSMOTIC RELATIONS IN MOLLUSCAN CONTRACTILE TISSUES

II. ISOLATED GILL PREPARATIONS FROM LAMELLIBRANCHS (*MYTILUS EDULIS* L., *OSTREA EDULIS* L., *ANODONTA CYGNEA* L.)

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The general problem initiating these researches was outlined in the previous paper (Pilgrim, 1953). The following pages describe the action of variations in osmotic pressure of the surrounding fluid on the gill cilia of three lamellibranch species of contrasting ecological distribution. Details of the media used are given in an Appendix to the previous paper.

MYTILUS EDULIS L.

The effect of osmotic pressure on ciliary activity was studied by Frédéricq (1922) who subjected *Mytilus* cilia to both hypo- and hypertonic sea water; he found that ciliary movement persisted 30 min. after immersion in 49–167% sea water,† but ceased in 37% and 185%; after 16 hr. there was still movement in 49–126% but none in 167%. Gray (1922) made a few remarks on the effect of osmotic pressure, noting that ‘... the stoppage of cilia in hypertonic solutions is brought about in an entirely different way to the stoppage in an acid solution, and it is therefore not surprising to find that the stoppage in hypertonic solutions is not influenced by the presence of hydroxyl ions...’. Wells, Ledingham & Gregory (1940) studied the action of hypotonic sea water on the cilia of various species of animals, including *M. edulis*. They found that sudden salinity changes produced ‘Shock Reactions’ followed by accommodation. They also noted that the different types of cilia on the *Mytilus* gill varied in sensitivity to osmotic pressure, and that disintegration of the gill occurred at salinities where most of the cilia were still beating; the latter observation may be of ecological significance, as disintegration may prevent the efficient working of the gill as much as immobilization of the cilia may do.

The writer’s experiments were undertaken (i) to extend the above observations into the hypertonic range, (ii) to determine if possible how far the effects of salinity change depend on the initial concentration, on the final concentration, or on the extent of the change, and (iii) to obtain comparative data on lamellibranchs of different ecological salinity relations.

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† Data from other workers have been recalculated to percentage sea water as in Paper I.

Methods

Single filaments were isolated by needles from the inner gill-lamellae and supported by glass threads on the underside of a slide over a capsule holding 40 ml. sea water; the activity of the various ciliated tracts was observed under a microscope with a $\frac{1}{8}$ in. objective. To change the bathing solution the slide was transferred to another capsule containing the fresh medium. Each experiment was conducted on filaments from a single gill and activity was rated normal (+++), reduced (++) , weak (+), nil (o), or hyperactive (+++ +). This method is subjective to some extent, in that in observing the accommodated beat after many hours it was difficult accurately to estimate slight changes from the initial beat; it was easy, however, to assess the relative frequency between cilia types after treatments since these observations were all made within a few minutes of each other. To obtain a more quantitative expression of the shock reaction in later experiments the times were noted at which the different cilia ceased and resumed beating.

The frontal, latero-frontal, food-groove and short abfrontal cilia retained their normal activity for at least 24 hr. after isolation in natural sea water; the long abfrontals were usually weaker after an hour and only feebly active in 24 hr. (though often quite vigorous if the capsule was shaken). The laterals were variable but often survived at their normal level of activity for 2-8 hr. and occasionally short lengths of the tracts appeared unaffected after 24 hr.—this clearly disagrees with Gray (1928, p. 99) who states that the laterals come to rest in sea water, a '... medium containing more magnesium than is present in the blood'. Analyses of *Mytilus* blood have since shown that the magnesium content is 97% that of sea water (Krogh, 1939, p. 56; *M. edulis*) and 96.5% (Robertson, 1950, personal communication; *M. galloprovincialis*), so that in this respect sea water appears to be a suitable bathing medium and Gray's results must be re-interpreted on some other basis. (It will be shown later that lateral cilia can withstand 150% sea water for several hours, though usually at a reduced level of activity.)

Survival of the different tracts was as satisfactory in artificial as in natural sea water; individual filaments showed slightly better survival in either natural or artificial sea water during the same experiment with no obvious 'preference' over the course of the investigation.

Results

(i) *The effect of hypertonic artificial sea water*

On sudden immersion in artificial sea water of more than 250% all cilia ceased beating within 1 min. and failed to recover; with less hypertonic solutions the different tracts showed greater or lesser degrees of salinity tolerance. The long-term results are expressed in Fig. 1, which disregards the temporary effects of change and shows the extent to which the cilia finally accommodated to the hypertonic medium and the limiting concentration in which only weakly active cilia (+) were found. By reading downwards the differentiating effect of any one concentration may be readily seen. In the case of the latero-frontals the individual cilia

appeared fully active in 150%, but the metachronal rhythm was often somewhat disturbed; a similar disturbance occurred with the laterals in all hypertonic media. (Gray, 1928, p. 128, remarks that 'The regulation which gives rise to the metachronal rhythm may quite likely be (and even probably is) independent of mechanical activity...')

The most resistant cilia are therefore those of the frontal, short abfrontal and food-groove tracts; the long abfrontals and latero-frontals are roughly equivalent, while the laterals are decidedly the most sensitive to an increase in osmotic pressure. This series conforms well with that described by Wells *et al.* (1940, p. 380) who found the same hierarchy of sensitivities on dilution.

Cilia	% sea water											
	20	30	40	50	75	100	125	150	175	200	225	250
Frontal	---	---	---	---	---	---	---	---	---	---	---	---
Latero-frontal	---	---	---	---	---	---	---	---	---	---	---	---
Lateral	---	---	---	---	---	---	---	---	---	---	---	---
Short abfrontal	---	---	---	---	---	---	---	---	---	---	---	---
Long abfrontal	---	---	---	---	---	---	---	---	---	---	---	---
Food groove	---	---	---	---	---	---	---	---	---	---	---	---

Fig. 1. *Mytilus* cilia; the extent to which accommodation was found in the different tracts. In Figs. 1, 2 and 4, thick line indicates normal activity (+++); thin line, weak activity (+); dotted lines, occasional preparations.

As was the case with heart preparations some types of cilia showed a shock reaction on first meeting a change of medium. In those concentrations in which full accommodation was later achieved, the cilia usually suffered no shock reaction or obvious reduction in activity (the long abfrontals are the chief exception to this, but they are an unsatisfactory type to study quantitatively since they are, even more than the laterals, so susceptible to mechanical stimuli); in the higher concentrations all types of cilia except short abfrontals slowed down before accommodating to lower levels of activity; this slowing down culminated in a temporary inhibition at the highest concentrations compatible with even weak activity.

(ii) *The relative importance of absolute concentration and of concentration change*

Having established that some types of cilia survived well in 150% sea water, the way was clear to conduct an examination of some of the fundamental factors producing the dilution effects. As was mentioned above, a few workers have described the effects of, and limits of survival in, hypotonic sea water, but their experiments were begun in 100% sea water. By running parallel experiments on filaments from

the same gill, commencing with some filaments accommodated in 150% as well as others in 100% artificial sea water, then diluting to various concentrations, it was thought possible to decide if the effective factor was either (1) the final concentration, or (2) the arithmetic difference between initial and final concentrations, or (3) the ratio final/initial concentration.

For example, a typical experiment included the preparations shown in Table 1:

Table 1

Capsule	Treatment on mounting	Reason
A	100% Plymouth tank sea water	Control
B	100% artificial sea water (A.S.W.)	Control
C	100% A.S.W. for 2 hr. then 30% A.S.W.	Standard for reference (E-J)
D	150% A.S.W.	Control
E	150% A.S.W. for 2 hr. then 30% A.S.W.	Effect of final concentration
F	150% A.S.W. for 2 hr. then 45% A.S.W.	Effect of ratio, final/initial concentrations
G	150% A.S.W. for 2 hr. then 80% A.S.W.	Effect of difference in concentrations
H	150% A.S.W. for 2 hr. then 35% A.S.W.	} Intermediate concentrations arbitrarily chosen
I	150% A.S.W. for 2 hr. then 40% A.S.W.	
J	150% A.S.W. for 2 hr. then 50% A.S.W.	

The most critical results were obtained on the shock reaction, as the time of onset and duration of inhibition could be determined with a stop-watch. After some practice, observations could be begun within 4 sec. of changing the slide to the fresh medium.

Frontal cilia subjected to a drop of 100 to 30% usually showed an inhibition lasting 3-4 min. The 150 to 80% treatment never caused inhibition, while 150 to 30% caused inhibition for periods of 4-14 min.; the preparations with an inhibition time most resembling the 100 to 30% were 150 to 45% and 150 to 40%. Similarly, 100-40% did not cause inhibition, whereas 150 to 40% did.

Latero-frontals were inhibited for much longer periods and, in the case of large drops in concentration, often failed to recover; it was clear that neither 150 to 80% nor 150 to 30% represented situations analogous to 100 to 30%. On the other hand, 150 to 75% had approximately the same effect as 100 to 50%, while 150 to 50% and 150 to 100% were quite distinct.

Short abfrontals were not stopped by either 100 to 30% or 150 to 30%; 100 to 25% did not cause inhibition, but 150 to 25% did for 10-25 min.

These observations show that while exact correspondence of inhibition times was not always obtained there was sufficiently close agreement to conclude that neither final concentration nor arithmetic difference between initial and final concentrations is of itself the effective factor determining the shock reaction behaviour; instead, the ratio 'final/initial' concentrations is decisive. The same conclusion appears to apply to the behaviour of the cilia after accommodation to the new medium. The activities of the cilia at 2-4 hr. after immersion in the hypotonic sea water were most differentiated in the extreme cases tested, namely the 100 to 30% drop; no preparation diluted from 150% matched the former exactly

in all activity ratings, but from a number of experiments it was clear that the most parallel treatment was 150 to 45%, that 150 to 80% was much more mild and 150 to 30% more severe in their effects; in other words, concentration changes approximating to the same 'final/initial' ratio led to quantitatively similar effects.

Wells *et al.* (1940, p. 383) noted that in some cases (the short abfrontal cilia of *Mytilus* as well as polychaete muscles) '...the inhibition evoked by downward change is preceded by a phase of excitation'; the phenomenon occurred after great changes (100 to 20%). It was here found present also in the frontal and food-groove cilia after the changes 100 to 50% or lower. Changes between 150 to 100% and 150 to 30%, inclusive, usually caused increased activity in these same three tracts for periods up to 4 hr., though in some of the extreme changes this phase was suppressed or was perhaps less than 4 sec. and so overlooked. The phenomenon is related, therefore, to a change in concentration rather than the final medium encountered. The effect does not seem to be related only to downward changes as it may occur in all three tracts after an increase in concentration; this compares with the observation of Wells & Ledingham (1940) who state that *Nereis diversicolor* body wall is at first excited on returning from hypotonic to normal sea water.

(iii) *Disintegration*

With extreme treatment numbers of ciliated cells became freed from the gill and even whole patches of the epithelium loosened. This was presumably due to a dissolution of the intercellular matrix (see Discussion), the ciliary activity helping to shake the cells free (particularly noticeable with latero-frontals). Such disintegration did not occur in 100% sea water after 24 hr. but was marked in hypertonic media; it was noted by Wells *et al.* in sea water less than 35%, so that it is a phenomenon found in concentrations at both ends of the physiological range; furthermore, in the present work on two changes of media the curious fact emerged that the extent of disintegration was related to the *ratio* of concentrations just as was ciliary activity (above). No attempt was made to establish an absolute scale of disintegration, but individual filaments during the same experiment could be easily compared.

OSTREA EDULIS L.

Despite a wealth of publication on feeding in oysters by ciliary currents very little has appeared on the physiology of the cilia themselves. Tomita (1934*a*) showed for *O. circumpecta* that mechanical (ciliary) activity of pieces of the gill was dependent on the concentration of the sea water medium; and also (1934*b*) that there was little effect on ciliary activity on increasing the hydrogen-ion concentration until pH 5.5 (approx.) when a sudden decline occurred.

Methods

The gills of *Ostrea* have much interfilamentar tissue making the isolation of single filaments impracticable. There is a further disadvantage with this animal in that the gills are heterorhabdic and plicate, the folding making it difficult to

examine cilia lining the water-pores. Preparations were made using pieces from all four gills since they are similar in their structure and ciliation (see Atkins, 1937, type B (1b)), and no difference was found in either their ease of preparation or their reactions to various media. The right valve of the shell and the right mantle lobe were removed and the gills divided into sections as in *Anodonta*;^{*} one lamella of each section was cut off, usually in small strips since the interfilamentar tissue of the grooves was seldom strong enough to hold all the filaments together during this operation. The sections of lamellae left *in situ*, however, were in about half of the attempts reasonably entire; they were cut off dorsally and mounted as for *Anodonta*, but using sea water. Unfortunately the preparation was fragile, tending easily to part along the grooves on stretching or, even if mounted satisfactorily, to undergo concertina-like movements by means of longitudinal muscles and so obliterate the water-pores or cover them and the grooves by folding. In most parts of the gill the apical and ordinary filaments are firmly united by a mass of tissue forming them into the shape of the plical folds, whereas the principal and two transitional filaments are chiefly united to this mass by small interfilamentar junctions and only sparsely by more substantial horizontal septa. This mass of tissue in the ridges of the plicae precluded clear observation on the cilia within the water-pores between apical and ordinary filaments so that attention was directed to the principal filament at the bottom of each groove. Providing these filaments had not been split or torn from their neighbours (transitionals) during mounting and were not torn subsequently as a result of muscular contractions, it was possible to observe lateral and latero-frontal† cilia along the principal filaments. Frontal‡ cilia were observed by the same device described for *Anodonta* though they were, perforce, those of the apical filaments; in some cases it was possible to observe the frontals of the ordinary filaments, but this could not be relied upon. On the whole the preparation, although the best obtained after many attempts, was not a very satisfactory one and only served to determine the range of salinity the three tracts could withstand; shock reactions were not studied since the tissue holds so much solution that concentration changes alongside the cilia might be slowed down and not be constant between preparations.

All three types of cilia were capable of maintaining normal activity in natural sea water for at least 24 hr. (in one case frontals showed undiminished activity 44 hr. after mounting in the capsule).

Results (see Fig. 2)

After sudden transfer to dilute sea water the frontals and latero-frontals survived as well and as long in 80 and 60% as their controls in 100%; in 40% survival was as long but often at a reduced level of activity, while all three types failed to recover in 20 and 10% sea water after 1–2 min. Laterals were the most sensitive cilia, their activity diminishing even in 80% while they were usually inhibited in 40%;

* More details are given for *Anodonta* as this yielded a more satisfactory preparation.

† Probably the 'anomalous latero-frontal cilia' of Atkins (1938).

‡ The 'coarse frontal cilia' of Atkins (1938), which beat ventralwards.

as in *Mytilus* these cilia were not very regular in their behaviour, mechanical disturbances setting them beating from an inactive state. A clue to this inconstancy was seen in scanning the whole preparation when it was evident that in the same piece of gill laterals of one principal filament might be beating while those of another were at rest. It was usual for the laterals from end to end of any one filament either to beat or not in all the species investigated, i.e. the discrepancy was between entire filaments and so was not detected in *Mytilus* using single filaments.

For experiments with hypertonic sea water, further controls were run in 100% artificial sea water; they gave results equivalent to those in natural sea water. All cilia failed to recover in 200% sea water except for very occasional quivering among the frontals after about an hour. Frontals survived in 125% as well as in 100% and in 150% with reduced vigour. Latero-frontals, and especially laterals, were affected by even 125%.

Cilia	% sea water									
	10	20	40	60	80	100	125	150	175	200
Frontal			---	---	---	---	---	---		
			---	---	---	---	---	---		
Latero-frontal			---	---	---	---	---	---		
			---	---	---	---	---	---		
Lateral			---	---	---	---	---	---		
			---	---	---	---	---	---		

Fig. 2. *Ostrea* cilia; the extent to which accommodation was found in the different tracts.

Swelling, followed by disintegration, occurred with all preparations placed in 20 and 10%; disintegration alone occurred in 200% and, to a slight extent, in 150%; these hypertonic media usually causing the tissue to become distinctly granular in appearance and less transparent. This was probably the result of dehydration of the cells with consequent disturbance in the physical state of the proteins and other colloids of the protoplasm. The swelling in hypotonic solution also resulted in constriction or even obliteration of the water-pores and other spaces between the filaments so that the laterals and latero-frontals were obstructed mechanically; as this swelling was most marked in sea water of less than 40% however, it is not considered that it affected the results for these tracts since frontals, shown to be the most hardy, had always ceased to beat in 20% without any indication of similar mechanical hindrance.

The salinity relations of *Ostrea* cilia appear from these results to resemble those of *Mytilus* cilia, except that the oyster cilia are less resistant to salinity change. This difference falls in line with that already found in the heart, and with the ecological relationships of the two species (Pilgrim, 1953).

ANODONTA CYGNEA L.

In contrast to the numerous published researches on other aspects of fresh-water mussel physiology, the study of their gill-cilia has been but little investigated. Ringer & Buxton (1885), in the course of the former's now classical studies on the importance of various ions to tissue viability, noted that gill-cilia of *Anodon* (= *Anodonta*) ceased to beat in distilled water within 24 hr., the cells having swollen and separated from the gill surface. Segerdahl (1922) found that cilia on isolated gill fragments kept beating up to 5 days in tap water and up to 9 days in 0.3% NaCl, though these were extreme cases. Zweibaum (1925) made tissue cultures from *Anodonta* gill, using diluted Frog-Ringer as a culture medium; the optimum concentration was R/3 ($\Delta = 0.15^{\circ}\text{C.}$) which maintained the preparations up to 63 days, while in $\Delta = 0.05^{\circ}\text{C.}$ and $\Delta = 0.38^{\circ}\text{C.}$ survivals averaged 16 days and 13 days respectively.

Methods

Owing to the complex eulamellibranchiate gill structure of *Anodonta* with the interfilar junctions stronger than the interlamellar it was not practicable to use single filaments as was done with *Mytilus*, so a preparation was devised as follows:

The animal was removed complete from its shell, pinned down, lying on one side, and the uppermost mantle lobe cut away to expose the gills of that side. The outer gill was pinned back and not used in these experiments since it has, in the female particularly, very thick interlamellar septa, while its use as a marsupium in the breeding season would have led to large numbers of embryos and glochidia being included in the preparations. The inner gill alone was used; it was divided dorso-ventrally with scissors at intervals of 1-1.5 cm.; each section in turn was now reflected to expose the dorsal margin of the ascending lamella which, for a considerable distance, is free of attachment to the visceral mass; this margin (rendered free where attached) was held up in broad-ended forceps while the interlamellar tissue was snipped a little at a time with fine scissors. Each section would contain two to four septa and towards the free (ventral) edge of the gill the lamellae could often be pulled apart once the initial cutting was completed dorsally; they usually parted along the food-groove yielding two similar pieces of tissue. The ventral 1-2 cm. only of each section was used and the piece was placed, frontal surface down, on a slide between two ridges of vaseline (or, better, 'Hard Grade' Vacuum Grease) with the ventral margin parallel to the ridges (Fig. 3). During the mounting the tissue was kept moist with the bathing medium. A firm glass thread, about 0.3 mm. diameter, was now placed to span between the grease ridges and parallel with the filaments to hold one side of the preparation in position; a second thread was similarly placed to hold the opposite side, but before pressing this thread well into the grease the tissue was stretched longitudinally with needles in order to open out the interfilar water-pores in the gill. The thread was then fixed in position and counteracted the tendency for the longitudinal gill-muscles to contract and so

obliterate the pores. This proved to be the crucial part of the technique; it was further essential to have one or preferably both of the glass threads at least 2 mm. from the edge of the tissue (see below).

The slide was then inverted over a capsule containing a suitable medium. Examination with the microscope gave a view of the outer surface of the gill; the terminal cilia could be seen at the ventral ends of each (half-) filament bordering the food-groove (itself slightly damaged and not clearly visible); within most of the water-pores were seen the latero-frontal and lateral cilia, the latter with a particularly striking metachronal rhythm. The frontal cilia were not easily detected over the bulk of the preparation as they lay parallel to the line of sight; further, their activity was very probably impeded by their being pressed against the slide,

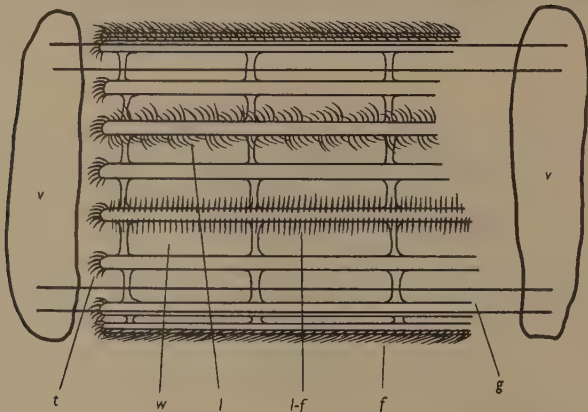


Fig. 3. Diagrammatic representation of a portion of *Anodonta* gill showing the method of mounting in the apparatus and the positions for viewing the several ciliated tracts. *f*, frontal cilia seen on filaments rolled over the glass thread; *g*, *l*, lateral cilia; *l-f*, latero-frontal cilia; *t*, terminal cilia; *v*, vaseline ridge; *w*, water-pore.

and by accumulations of mucus exuded following this mechanical stimulation. Instead, they were visible where the free 2 mm. of the tissue curled over the glass threads; by focusing it was possible to see, at their several levels, the frontal tracts of three to four filaments in profile; in this position the frontal cilia were not subject to pressure against the slide nor to sheets of entangling mucus.

For a bathing medium 4% sea water was used; tap water, although the medium applied without adverse effect to the whole animal in the aquarium, failed to maintain normal ciliary activity for the same length of time; this was understood to imply that the medium bathing the *isolated* gill tissue enters the vessels normally containing blood, so that it is necessary to bathe with a solution imitating the blood in ionic strength. Shock reactions were not investigated for the same reason as in *Ostrea*; the water spaces are even more extensive in *Anodonta* gill.

In 4% sea water or in artificial *Anodonta* blood, the whole preparation remained

healthy in appearance with all tracts of cilia observed beating vigorously for at least 24 hr.; in two instances up to 40 and 49 hr. respectively.

Results (see Fig. 4)

In 2% sea water the laterals sometimes, and the other three types always, maintained normal activity; in 1% latero-frontals did not always beat as vigorously as in 4%. Similarly, in the case of artificial blood comparably diluted, there was the same falling-off of activity in laterals and latero-frontals. Frontals and terminals sometimes beat normally in (London) tap water but generally all activity was quickly reduced and laterals inhibited.

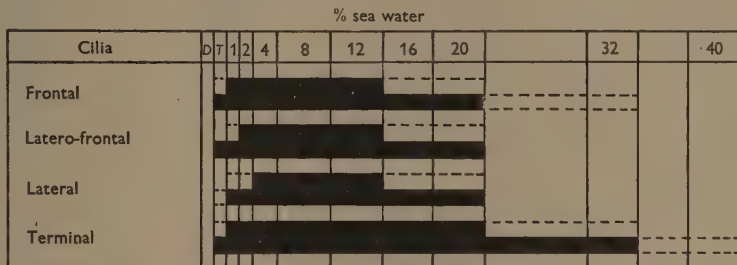


Fig. 4. *Anodonta* cilia; the extent to which accommodation was found in the different tracts. D, distilled water; T, tap water.

A few frontal and terminal cilia maintained a weak beat up to 2 hr. in distilled water and in $M/400\text{-NaHCO}_3$, but in the majority of cases all cilia ceased beating after 1-2 min. in these fluids. Although the distilled water was rather acid it is not thought this was the main contributory factor to inhibit the beat since $M/400\text{-NaHCO}_3$ failed to support even weak activity for more than a few minutes.

All cilia were apparently unaffected by 8-12% sea water, terminals and often others beat actively in 16-20%, while frontals sometimes beat feebly in 32% and terminals in 40%. Terminal cilia in hypertonic media tended to stand out straight from the surface and beat very vigorously, rather than bend in the direction of beat. The reaction to comparable strengths of artificial blood was in each tract equivalent in respect to survival and activity.

Slight swelling occurred in 1% sea water but no disintegration within 24 hr.; in tap water swelling was more pronounced and may have interfered with the lateral cilia as the water-pores narrowed. Disintegration was most striking, following extensive swelling, in distilled water, the tissue falling to pieces if shaken, in an hour or less. Raising the pH by using $M/400\text{-NaHCO}_3$ failed to prevent this deterioration or even to retard it noticeably. In hypertonic media there was disintegration without swelling, becoming evident in 16% sea water and more extensive as concentration increased. It is interesting to note that disintegration was present in hypertonic artificial blood to about the same extent as in the corresponding sea water concentrations; swelling in diluted media was, however, a little less marked in the former.

DISCUSSION

The frontal cilia (all three species), short abfrontals (*Mytilus*) and terminals (*Anodonta*) are excellent for experimentation and withstand considerable degrees of concentration and dilution of the media. The latero-frontals and especially laterals, of all three, however, do not survive very long in the isolated gill even in their normal media; it is suggested that unequivocal results can be obtained from these tracts only with extreme caution. Some disadvantages of these two tracts are pointed out in earlier sections; in *Ostrea* and *Anodonta* it was found also that a length of lateral ciliated tract beating normally, with its precise metachronal rhythm, might suddenly cease, the cilia remaining stationary at the end of the effective beat; this was seen on numerous occasions in 100 and 4% sea water respectively and always *all cilia appeared to cease together*, but resumption some seconds later progressed slowly along the tract. There was no obvious reason for the cessation, and a sharp blow on the bench did not produce it. Lucas (1931) found laterals and latero-frontals in several species to behave in this way both *in vivo* and *in vitro*; he stated there was no nerve supply down the gill filaments so the central nervous system cannot be responsible for the effect. Some degree of intercellular co-ordination, however, must be present in tracts with such highly organized rhythm, and it is presumably upon this mechanism that there acts the stimulus for cessation.

The differing sensitivities of various cilia types found by Wells *et al.* (1940) on dilution in *Mytilus* are shown to hold for hypertonic sea water in *Mytilus*, and for both hypo- and hypertonic media in *Ostrea* and *Anodonta*.

In the previous paper (Pilgrim, 1953), it was pointed out that a correlation appeared to exist between blood concentration and the whole metabolism of these animals, particularly their heart rate. A curious exception to this correlation is in the rate of beat of the cilia; in all three species the gill cilia of corresponding types beat at approximately the same rate in their proper media. It is instructive to compare the ecological ranges and the sea water concentrations in which heart preparations beat well (see Table 2, Pilgrim, 1953), with corresponding values for frontal cilia (the most reliable ones occurring in the three species used). The ranges, in percentage sea water, are, respectively, *Mytilus* 45-100, 40-160, 40-150; *Ostrea* 70-100, 70-120, 40-125; *Anodonta* fresh water-*c.* 14, 2-*c.* 24, 1-12. The frontal cilia of *Mytilus* and of *Ostrea* are, like their hearts, capable of normal activity over salinity ranges greater than those to which the whole animals are subjected in natural conditions. Further, the heart and cilia tolerances within each of these two species are similar; this is to be expected in poikilosmotic animals. *Anodonta* heart will not beat when isolated in fresh (tap) water, and this is consistent with the homoiosmotic nature of the animal; the frontal cilia, however, show a reduced activity when isolated in tap water. In the case of the three heart muscles it is reasonable to postulate that rate of beat is proportional to the concentration of one or more ions in the immediate environment, i.e. blood and pericardial fluid, which are almost identical, and together vary with changes in outer environment. Much

the same ionic media bathe both sides of the ciliated cells on the gills of the marine species over a wide range of salinities, but in *Anodonta* these cells are, *in vivo*, constantly subjected to pond water on one side and, on the other, to the blood which is 10–20 times as concentrated. Despite their very dilute bathing media the cells can evidently maintain a sufficiently high concentration of the necessary ion(s) to support vigorous ciliary activity. Probably they are much more dependent on blood concentrations and are ionically somewhat insulated from the fresh water; that they are not completely unaffected by external media is shown by their inability to function in distilled water, or in sea water above 32%, in which conditions the cells must be unable to maintain their internal ionic constitution in the face of such extreme concentration gradients. The activity of the cilia over wide ranges of salinity lends further support to Needham's suggestion (1930) that ecological distribution is related to factors in the life history of the species rather than to the behaviour of the adult.

Disintegration is shown to occur in *Mytilus* (below 35% and above 150% sea water), *Ostrea* (below 30% and above 150%) and *Anodonta* (in tap water and above 12% sea water); in each species swelling accompanies or precedes the effect in hypotonic but not in hypertonic media. A different explanation is suggested in the two situations:

(a) Gray (1931) showed that the stability of the intercellular matrix of *Mytilus* gill was dependent very largely on the magnesium ion but also on the other major cations; on dilution, then, there are probably insufficient of the requisite ion(s) to stabilize the matrix which softens and frees the cells; they free themselves all the more easily if still beating. Conditions for stability are markedly different at different pH (Gray), but this was held constant here. In *Anodonta* blood and natural waters magnesium is scarce and possibly calcium is the more critical ion, yet disintegration was approximately equal in 1% sea water and in $\frac{1}{4}$ *Anodonta* 'blood'; calcium is about 18x as concentrated in the latter.

(b) In hypertonic media disintegration was usually accompanied by a granular appearance in the tissue; the latter is attributed to dehydration of the cells with consequent denaturation of the protoplasmic proteins. Disintegration itself, it is here suggested, is due to the cells shrinking and loosening from the matrix and from each other; if still beating they assist in freeing themselves.

The added loosening effect of the still-active cells gave rise to rather anomalous results on occasion; disintegration was more apparent in 150% sea water (*Mytilus*) than in 200%, presumably the greater ciliary activity freeing the larger number of cells.

SUMMARY

1. Isolated preparations are described from the gill epithelium of three species of Lamellibranchs; sea water was found to be a suitable medium for *Mytilus* and *Ostrea* and 4% sea water for *Anodonta*.

2. Sudden changes in concentration of the media led to 'Shock Reactions' in several types of cilia. The extent of the shock reaction following dilution was shown

to be related to the ratio of the concentrations of the media before and after the change, rather than to either of these values separately.

3. In all three species the range of salinity compatible with mechanical activity varied with the type of cilium, being widest in the frontals, narrowest in the laterals, and intermediate in the other tracts.

4. Disintegration of the epithelia is found in all three species in both hyper- and hypotonic media, with accompanying swelling in the latter.

The work described in this and the previous paper was done in the Department of Zoology, University College, London.

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THE TEMPERATURE OF WOODLICE IN THE SUN

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INTRODUCTION

Information on the significance of solar radiation in the ecology of arthropods, though still very scarce, is beginning to accumulate. Most of the work refers to insects, and in many cases insolation appears to be advantageous, by accelerating development and in other ways. The subject has been reviewed by Gunn (1942) and Uvarov (1948). Apart from insects and spiders, most terrestrial arthropods are cryptozoic. But this is not invariably so: several species of woodlice, for example, are sometimes exposed to direct sunlight, and, for them, such exposure would appear to be more dangerous, in view of the relative permeability of their integument and of their comparatively low thermal death point (Edney, 1951*a*). It is also known that, under laboratory conditions, the internal temperature of woodlice is lower than that of the surrounding air if the latter is dry (Edney, 1951*b*), and this raises the question of the combined effects of radiation and evaporation when woodlice are exposed in the field to direct sunlight. The present work was undertaken in an attempt to measure these effects, and to approach the problem of their significance as ecological factors.

MATERIALS AND METHODS

The greater part of the work was carried out on *Ligia oceanica* Linn., which is a littoral animal; but comparative measurements were made on *Oniscus asellus* Linn., *Porcellio scaber* Latr. and *Armadillidium vulgare* Latr. The cockroach, *Blatta orientalis* Linn., was also used for comparative purposes. This insect does not, of course, normally occur in sunlight—it was used because it is similar in shape and size to *Ligia* but has a relatively impermeable cuticle.

Before use experimental animals had continuous access to food and water, so that they were presumably all in a similar physiological condition. No animals preparing for, or recovering from, a moult were used, neither were gravid females. So far as possible, animals of a similar size were used in each experiment. Only female specimens of *Blatta* were used, to avoid the complication of wings, which are present in males and cover the abdomen.

The work on *Ligia* was carried out mainly at Dale Fort, Pembrokeshire, during the summers of 1951 and 1952; that on the other species was done at Birmingham in the summer of 1952.

Thermocouples, as described previously (Edney, 1951*b*), were used to measure internal temperatures, and air and ground temperatures. ('Ground' is used to

describe the rock, concrete, slate or wooden surfaces upon which animals were exposed—the material used is specified in each experiment.) The thermocouples were connected by a multi-way bipole switch to a galvanometer. This arrangement allowed rapid reading of different thermocouples in succession. A sensitivity of $0.7 \text{ cm./}^{\circ} \text{C.}$ was obtained, and the apparatus could be relied upon to introduce an error no greater than $\pm 0.2^{\circ} \text{C.}$

When a living animal was exposed, it was held by a loop of cotton fixed to the ground by plasticene. This allowed a free circulation of air, and the flexibility of the fine thermocouple wires allowed a small degree of movement.

Now the equilibrium temperature reached is the result of a complex interplay of a number of factors which are likely to fluctuate rapidly during measurements made in the field, and this raises the question as to the best method for recording such temperatures. Short-period fluctuations were in fact often encountered, and the problem was investigated by taking readings from a number of pairs of animals (one pair at a time) as rapidly as possible (about once every 6 sec.) for 3 min. periods. One member of each pair was living, the other dry. From one such series of readings, obtained with *Oniscus*, it appears that the dry *Oniscus* is consistently about 1.5°C. warmer than the living specimen, but if readings had been taken less frequently, once every 30 sec. from each animal alternately, it so happens that three successive readings from the living *Oniscus* would have been higher than those from the dry specimen, thus giving an entirely false picture.

This particular set of readings shows a good deal more fluctuation than most other comparable sets did: it was chosen to illustrate the greatest possible error. Furthermore, it is unlikely that readings taken at random would remain in phase with temperature fluctuation for long, but the experiment shows that results must be interpreted with caution: in particular, little significance can be attached to very short period temperature differences, or to the fact that curves running close to one another occasionally cross. It is clear that continuous temperature recording would be the ideal technique to use, but in the absence of such apparatus a compromise must be reached, and if readings are taken frequently enough it is still possible to obtain records which furnish all the information necessary for the present purpose. The usual procedure, therefore, was to take several series of readings at 10 sec. intervals for periods of about 3 min. or more. Intervals between successive series of readings depended upon the amount of fluctuation encountered in the previous series (the greater the fluctuation, the shorter the interval), but were usually less than 5 min. (except during a long period of shade). In plotting the results, when the fluctuation is slight, the mean of each series of readings from each thermocouple is taken to represent the temperature of that thermocouple half-way through the series of readings; but when fluctuation is greater, individual readings are plotted and the readings follow one another at 10 sec. intervals (see, for instance, fig. 4, where seven thermocouples were in use, and where this method was followed throughout the exposure). The points obtained are joined by straight lines, but it follows from what has been said above that these should not be taken to represent continuous temperature changes.

Humidity was measured by a small electrical hygrometer (Edney, 1953) about the size of a full-grown *Porcellio*. This was placed a few millimetres above the ground, just to windward of the animals, but shaded from direct sunlight. The elements used could be relied upon to read relative humidity to $\pm 2\%$, but the use of the element in the shade while the animals were in the sun needs a little justification. Air passing over insolated ground is warmed, and its R.H. is therefore lower than that of air away from the ground. The animals are exposed to this warm air, and the fact that it passes over shaded ground for a fraction of a second near the hygrometer will not affect its temperature. Thus the element will show the humidity of a sample of air similar to that which passes over the animals. To expose the hygrometer itself to the sun would raise its temperature considerably, and thus lead to greater error, for the hygrometer depends upon the resistance of glass yarn impregnated with CaCl_2 , and this resistance varies with temperature as well as with humidity.

Wind speed was estimated approximately on the Beaufort scale. It became clear from the analysis of the present results that wind velocity is of great importance as a factor in determining equilibrium temperature, and in future work it would be very desirable to measure it accurately, as near to the animal as possible.

EXPERIMENTS

(a) *The metabolic heat of Ligia*

A preliminary experiment was carried out to find whether living *Ligia* would produce sufficient metabolic heat to affect significantly the interpretation of the main results. It was known that the effect, if any, would be small, and since only differences in temperature between a living and a dead animal were required, a sensitive thermocouple, without a reference junction, was used. Both junctions were very fine: one was inserted into the rectum of a living *Ligia*, the other into an animal which had been killed an hour previously and kept in saturated air to prevent evaporation. The thermocouples were carefully zeroed before and after the experiment, and they could be relied upon to indicate differences of temperature down to 0.1°C . The animals were suspended freely in nearly still air during the experiment.

The results showed that, after settling down, the temperature of the living *Ligia* was $0.4 \pm 0.1^\circ \text{C}$. above that of the dead specimen, and remained so for an hour. After this time, the living specimen was killed, without removing the thermocouple, by introducing it into a bottle of chloroform vapour. After settling down again, the temperature difference had fallen to $0.1 \pm 0.1^\circ \text{C}$.

This increase of 0.4°C . due to metabolism was obtained in laboratory conditions: its significance in field exposures will be discussed below (p. 343).

(b) *The validity of using dry specimens as controls*

Before proceeding to the main results, there is one further point to be considered. In order to estimate the effect of evaporation alone upon the temperature of the living animal, a dead, dry animal of the same size was to be exposed in the same environment. That the *rate* of change of temperature of a dry woodlouse will be greater

than that of a living one is to be expected, owing to its smaller volume and thermal capacity; but in theory, since the rate of transfer of heat by conduction, convection and evaporation depends upon surface area, change of volume should not affect the final *equilibrium* temperature. It might, however, be suggested that a thoroughly dry woodlouse has a significantly different shape from that of a living specimen: it might, for instance, present a greater surface to the wind for convection. It was therefore decided to investigate the problem experimentally. For this purpose a living *Ligia* was dipped into a solution of beeswax in chloroform. This killed the animal and deposited a thin layer of wax over its surface. The dipping process was repeated twice. The waxed *Ligia* was then exposed in the sun upon dark slate together with a living and a dry specimen.

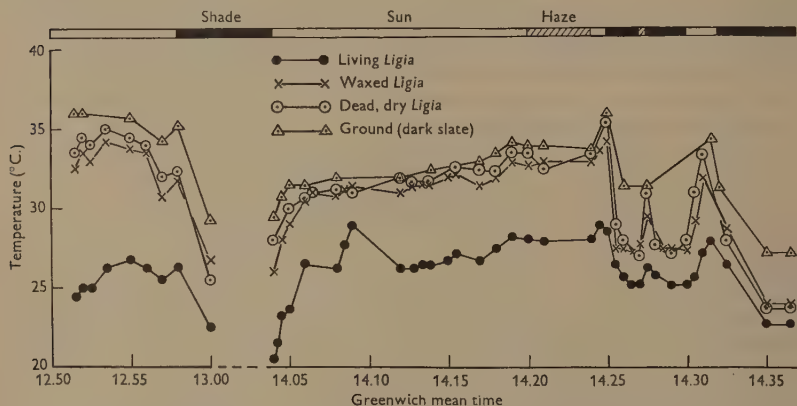


Fig. 1. The internal temperature of a living *Ligia* compared with that of a dead, dry specimen, and of a waxed specimen, during insolation upon dark slate. R.H. 52-60%; wind speed *c.* 200 cm./sec.

The results are shown in Fig. 1 and they are reasonably conclusive, for the temperature of the waxed specimen is throughout very close to that of the dry one (usually within $0.5^{\circ}\text{C}.$), and both are a good deal higher than that of the living animal. That the waxed specimen is for the most part slightly cooler than the dry specimen is probably due to incomplete waxing, for it is very difficult to be sure of depositing a continuous layer over the whole surface. However, the temperatures were considered sufficiently close to warrant the use of dried specimens as controls.

(c) *The effect of radiation and evaporation on the internal temperature of Ligia*

Experiments were carried out on *Ligia* at Dale Fort, both on a concrete quay and on Red Sandstone rock. In each of these experiments, three specimens were used, one was alive, the second was freshly killed and still moist, and the third had been killed and thoroughly dried. They were exposed horizontally, side by side, with

the dry specimen to windward, and, so far as possible, broadside to the sun. Experiments were also made in which the animals were mounted upon a movable wooden block, which could be taken from the shade and placed in the sun without disturbing the thermocouples. Air temperature was measured 5 mm. above the surface of the ground, and the ground temperature itself was measured by a thermocouple in close contact with it. This thermocouple was fine enough not to be affected significantly by radiation, but the measure cannot entirely be relied upon, for at least the upper half of the junction must have been exposed to the air. Exposure on an insulated rock or stone meant exposing the animals to higher temperatures than exposure on a wooden block moved from shade to sun, for the block itself took some time to warm up.

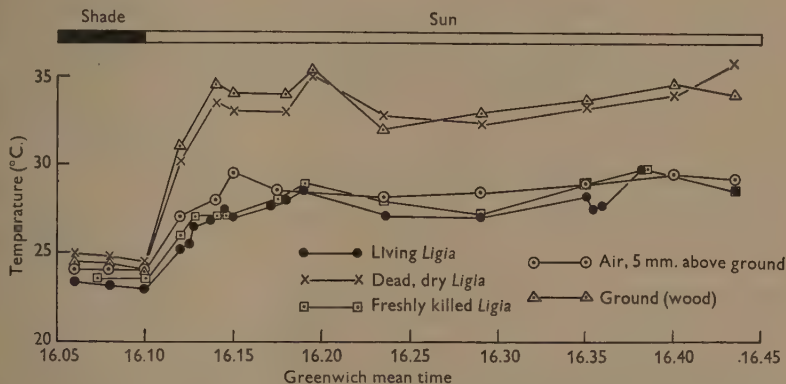


Fig. 2. The internal temperatures of living, freshly killed, and dead, dry *Ligia*, compared with the air and ground temperatures during insolation upon wood (light deal). 25 July 1952. R.H. 45-52%; wind speed *c.* 200 cm./sec.

The temperature curves obtained in all these experiments show the same general picture, though they differ in ways which will be discussed immediately. A typical set of curves which refers to an exposure on a wooden block, moved from shade to sun, is shown in Fig. 2. The readings commence after allowing temperature changes due to handling, etc., to settle down. During the first 5 min. (exposure in the shade), all the curves are close together, and the living *Ligia* is about 1.5° C. cooler than the dry specimen. As soon as the preparation is placed in the sun, all the temperatures begin to rise: those of the ground and of the dry *Ligia* by approximately the same amount, those of the living and freshly killed *Ligia*, and of the air, to a lesser extent. After the first 5 min. in the sun, the curves remain steadier until the readings cease 30 min. later.

During the period of comparative stability from 16.23 to 16.35 hr. the temperature of the ground and the dry *Ligia* remain within 0.5° C. of each other (that of *Ligia* usually being slightly the lower of the two) and about 5-6° C. above that of

the living and freshly killed specimens. This difference in temperature can be ascribed entirely to evaporation of water from the moist animals. The air temperature during this period was from 0 to 1° C. above that of the moist animals. In the sun the temperature of the living *Ligia* was from 4 to 5° C. higher than it was in the shade a few minutes previously, and this is a result, directly and indirectly, of exposing the animal and its immediate environment to increased radiation.

The quantitative aspect of the factors involved in this situation is discussed below (p. 344); for the present we may examine other records for *Ligia* (some of which are summarized in Table 1) to see how they vary from the set chosen for illustration in Fig. 2.

All the records show a higher temperature for dry *Ligia* than for the living or freshly killed specimens, but the amount of this difference varies both between one set and another, and from time to time within the same set. The greatest difference which remained approximately constant for 10 min. was 9° C., though differences greater than this were recorded for short intervals (but such records may be spurious, see above, p. 332). An early record, in which a difference of about 7° C. was maintained for 20 min., has been briefly reported elsewhere (Edney, 1952), but the majority of curves show temperature differences in the regions of 5–6° C. The amount of the difference is of course dependent upon various climatic factors, and these are also discussed below.

Short-period fluctuations both in general temperature level and in temperature differences are found in all records: these are due largely to variations in wind speed. Such fluctuations are also caused by variation in radiation: where cloud or haze intervenes, all temperatures drop, but some to a greater extent than others.

The relation between dry *Ligia* and ground temperature also varies from one record to another. The curves shown in Fig. 2 are fairly typical, but records have been obtained in which the dry *Ligia* was warmer than the ground surface; in others again, *Ligia* was several degrees cooler than the ground. These differences will depend upon a number of factors, the chief of which will be wind speed and the nature of the ground surface so far as absorption and reflexion of radiation are concerned. Lastly, the air temperature, while always lower than that of the isolated dry *Ligia* or the ground, may be either higher or lower than that of living *Ligia*.

(d) *The surface temperature of Ligia in the sun*

During the course of some of the exposures of *Ligia* upon rock at Dale Fort, an attempt was made to measure the surface temperature of the integument. A thermojunction was held in close contact with one of the tergites against the posterior margin of the preceding tergite. The measure is not perfectly accurate, but provides a reasonably good approximation. The surface temperatures of living and of freshly killed (still moist) specimens always corresponded within 0.5° C., and they were always below the rectal temperatures of the same animals, even when the rectal temperatures were rising. This is a somewhat surprising observation until it is remembered that surface temperatures were taken on the tergites: the ventral surface may have been very much warmer owing to conduction and radiation from

the hot rock upon which the animals were mounted. After settling down, surface temperatures were from 1 to 3° C. below rectal; they were very sensitive to changes in wind speed, and fluctuated considerably, even when the rectal temperatures were fairly constant. The surface temperatures of dry specimens also fluctuated, but they usually remained within 1° C. above or below the rectal temperature. In view of the difficulty of measuring surface temperatures accurately, and of their greater fluctuation, only rectal temperatures were measured in most experiments, and rectal temperature is used to represent the temperature of the animal in the analyses below.

Table 1. *Internal temperatures of various species of woodlice exposed to solar radiation, measured during comparatively stable periods*

Species	Living animal (° C.)	Dead, dry animal (° C.)	Dead, fresh animal (° C.)	Ground (° C.)	Air 5 mm. above ground (° C.)	Relative humidity (%)	Wind speed (cm. sec.)	Time (G.M.T.)	Date	Nature of ground
<i>Ligia oceanica</i>	28	34.5	28	35	21	67	50	14.15	14. viii. 51	Rock*
	30	37.5	—	39	30.5	30	300	14.25	24. vii. 52	Stone*
	29	37	27	37	30	45	450	15.00	24. vii. 52	Wood*
	27	32.5	27.5	33	28.5	48	200	16.30	25. vii. 52	Wood
	36	41	—	45	33	52	300	12.30	26. vii. 52	Stone
	27	31	—	30	24.5	68	150	14.25	5. vii. 52	Rock
	—	—	—	—	—	—	—	—	—	—
<i>Oniscus asellus</i>	39	43	39.5	49	31	40	300	13.30	22. vii. 52	Stone
	35	40	35	45	31	45	300	14.20	22. vii. 52	Stone
	36	37.5	—	37.5	32	47	100	15.20	22. vii. 52	Wood
	33.5	35	33	36	31.5	50	150	16.05	22. vii. 52	Wood
	—	—	—	—	—	—	—	—	—	—
<i>Porcellio scaber</i>	31	32.5	31	35	27	43	250	9.10	24. vii. 52	Wood
	35	36.5	35	38	32	41	200	11.20	24. vii. 52	Wood
	37.5	39	—	41	32	35	100	13.10	24. vii. 52	Stone
<i>Armadillidium vulgare</i>	28.5	29	29	30	27.5	59	300	15.12	2. viii. 52	Wood
	34.5	36	34	37.5	33.5	53	300	15.50	2. viii. 52	Wood

* Rock: weathered Old Red Sandstone; stone: dark slate; wood: smooth, light deal.

(e) *The internal temperature of other species of woodlice*

The rate at which different species of woodlice lose water by evaporation corresponds, so far as order is concerned, with their temperature depression under laboratory conditions (Edney, 1951*b*). Measurements were now made to find whether there are comparable differences when the animals are exposed to radiation in the field. Exposures of *Oniscus asellus*, *Porcellio scaber* and of *Armadillidium vulgare* were made on insulated stone and on movable wooden blocks. These experiments were all carried out on the laboratory roof at Birmingham, and the results are summarized in Table 1. The capacity to evaporate water, and thus reduce temperature, lasts a remarkably long time: one record, for instance, shows that *Porcellio*, placed in the sun at 11.00 hr., is at 12.30 hr., after 1½ hr. nearly continuous exposure to sunlight, still capable of lowering its temperature by 1.5° C. compared with a dry specimen.

It is noticeable that, compared with living *Ligia*, the temperature of living

Oniscus and other species rises very rapidly when the animals are subjected to radiation. This is due to their smaller size and consequently larger surface area/volume ratio. There is also less difference between the temperature of living and corresponding dead specimens of these smaller species than there is in the case of *Ligia*, but this is due to the fact that, per unit area, water evaporates less rapidly from them than from *Ligia*. There is no significant difference between the temperatures of living and freshly killed specimens of any species.

(f) *The internal temperature of Blatta orientalis compared with that of Ligia*

A living female *Blatta* was exposed together with living and dead *Ligia*. Since *Blatta* has a relatively impermeable wax in the epicuticle it might be expected to show a somewhat higher temperature than *Ligia*, although it is approximately the

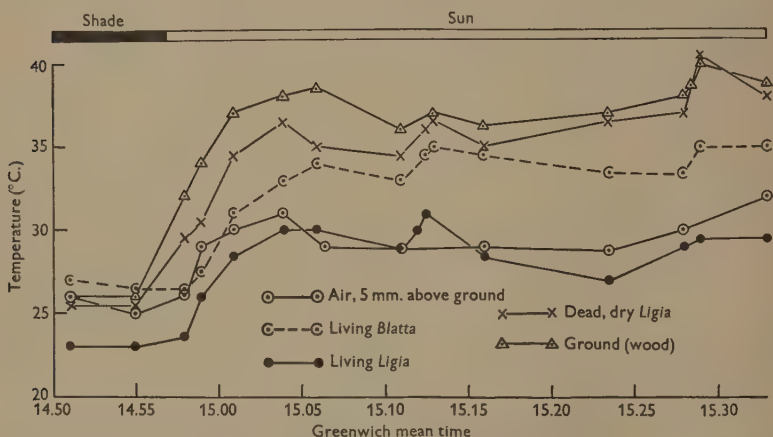


Fig. 3. The internal temperature of a living cockroach (*Blatta orientalis*), compared with that of a living and of a dead, dry *Ligia*, during insolation upon wood (light deal). 24 July 1952. R.H. 39-45 %; wind speed *c.* 500 cm./sec.

same size and weight. The results are shown in Fig. 3, and it is clear that the temperature of *Blatta*, though lower than that of the dead *Ligia* and of the ground, is nevertheless a good deal higher than that of the living *Ligia*; the difference, during comparatively stable periods, varies between 4 and 6° C. During most of this exposure, the temperature of *Blatta* is well above 30° C., and in the region of its 'critical temperature' (Wigglesworth, 1945). This may account for the fact that the temperature of *Blatta* is depressed at all, for, as Parry (1951) shows, the evaporation of water from insects below their critical temperature is, on theoretical grounds, insufficient to affect the equilibrium temperature significantly.

(g) *Comparative measurements of internal temperature depression*

The exposures so far considered were made at different times, and since conditions were different there is little point in comparing temperature depressions. In order to make such a comparison the species must all be exposed at the same time. This was done in two exposures, and substantially similar records were obtained, one of which is shown in Fig. 4. The air temperature was taken as usual, but it is not shown in the figure (which is already complex enough) for it followed that of *Ligia* quite closely. To facilitate handling and mounting, all the animals used in these exposures were killed immediately before exposure.

Of the woodlice, *Ligia* shows the greatest depression (taken here as depression from ground temperature): during the comparatively steady period from 14.20 to 14.30 hr. this was about 8.0°C . It tends to decrease later on, presumably because water becomes less readily available for evaporation as the animal dries. *Oniscus* shows a depression, during the same period, of about $4\text{--}5^{\circ}\text{C}$., and *Porcellio* is lower than ground temperature by some $2\text{--}3^{\circ}\text{C}$. These results correspond, at least so far as order is concerned, with temperature depression found in the laboratory, but *Armadillidium* shows a depression which is throughout the exposure nearly as great as that of *Oniscus*—a result which is at first sight disturbing, for the rate of evaporation of water from *Armadillidium* is known to be considerably lower than that from *Oniscus*. The explanation is probably twofold: in the first place, the surface of *Armadillidium* is much more shiny than that of *Oniscus*, so that more radiation may be reflected and less absorbed; secondly, the dorsal surface of *Armadillidium* is strongly rounded, while that of *Oniscus* is depressed, so that *Oniscus* will be subjected to a greater mean radiation intensity per unit area than *Armadillidium*.^{*} These two factors may well combine to counteract the effect of a greater rate of evaporation from *Oniscus*. The observation serves as a reminder that internal temperatures established in the laboratory, where radiation is of little account, are no guide to the situation under natural conditions in the field.

A cockroach was exposed at the same time as the woodlice, and readings of its temperature are inserted in Fig. 4. In general, the points lie between those referring to *Porcellio* and *Oniscus*, occasionally running above the former. In the laboratory, too, at comparable temperatures around the critical point, *Blatta* shows a somewhat greater depression than *Porcellio* (Edney, 1951b).

(h) *Observations and measurements in the natural habitat*

The results reported above were obtained in the field in the sense that animals were exposed out of doors, but it cannot be claimed that the conditions were always within the normal habitat range. (This can certainly be claimed, however, for one or two experiments with *Ligia* during exposure on rock at Dale Fort, for wild *Ligia* actually wandered across the experimental arena.) Further measurements were therefore made of conditions in the presence of wild woodlice. They are

^{*} This is true provided the elevation of the sun is greater than about 28° ; in the exposure being considered the sun was about 45° above the horizon.

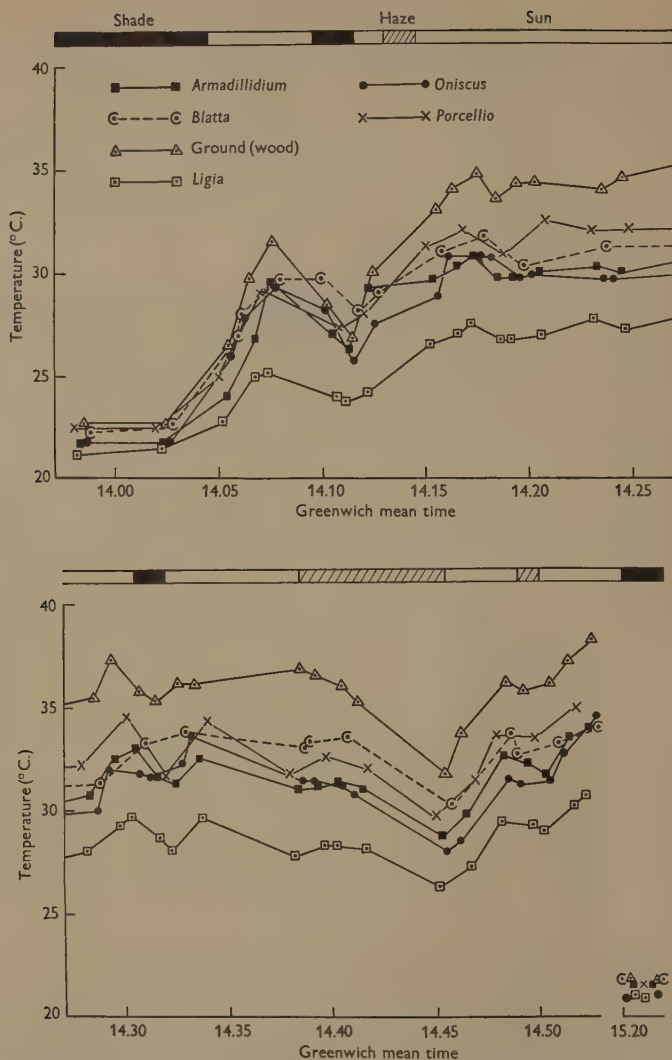


Fig. 4. The internal temperatures of various species of woodlice and the cockroach *Blatta*, exposed simultaneously to insolation, upon wood (light deal). Air temperature 5 mm. above the ground followed that of the living *Ligia* closely. 14 August 1952. R.H. 55-63 %; wind speed c. 50-100 cm./sec.

reported here, but they are incomplete, and further work is needed if the ecological suggestions put forward are to be tested.

Measurements of temperature were made on the vertical face of a concrete quay at Dale Fort, exposed to direct insolation, at 16.30 hr. on 29 June 1952. *Porcellio scaber* and *Ligia oceanica* were present. Temperatures of 36.1–38.6° C. were recorded on the surface of this quay where *Porcellio* were walking. Lower down, where *Ligia* were walking, the surface temperature was in the neighbourhood of 32° C. At the same time the temperature of the air 5.0 mm. above the surface was from 32 to 35° C. (near *Porcellio*) and 28 to 32° C. near *Ligia*. The temperature in the deep crevices of the quay, and below the shingle at the foot, was much lower from 21 to 25° C. A favourite place for *Ligia* to congregate and remain for several hours was a shallow depression in the vertical surface of the quay, where water was slowly oozing out. The temperature of the surface here was 30.6° C. Most of the *Porcellio* and all the *Ligia* seen on this occasion were immature specimens, and the observations suggest that, by exposing themselves to insolation, they were utilizing a favourable microclimate in which development would be accelerated. Loss of water by evaporation was no problem, for it was on the spot or close at hand.

Further measurements were made in a sheltered and insulated bay, just above high-tide mark, where the shingle gave place to Old Red Sandstone cliffs. *Ligia* and *Porcellio* were often to be seen walking over the surface of these rocks in direct sunlight, and large colonies were present under shingle together with decaying organic matter. The humidity and temperature were measured in these microclimates, and a typical set of observations, made at 14.00 hr. G.M.T. during a sunny day in August 1951, is shown in Fig. 5. Air under the shingle was nearly (perhaps quite) saturated with water vapour and its temperature was as high as 30° C. owing to insolation of the stones. The internal temperature of *Ligia* under the stones was also 30° C., which approaches the lethal temperature for 1 hr. exposures in saturated air (32.5° C.). Above the shingle on the rock surface, temperatures varied considerably from place to place, the highest recorded was 38° C., but small crevices, changes in angle, colour, etc., provided a wide range of conditions over the area. The temperature of *Ligia* was 26° C. when walking over the surface of rock at 34° C. The air temperature 2 cm. above the rock was 20° C. and its relative humidity from 60 to 70%.

It seems not unlikely that in such circumstances the animals are caused to emerge from below the shingle to a region where humidity and air temperature are both lower, so that they can lose heat by evaporation and convection, and thus lower their temperature even though they are exposed to direct sunlight. Evaporation of water will be severe, and the animals will be forced to return to a region of high humidity unless they find a cool, moist microclimate in the crevices of the rocks. No detailed records of the wanderings of *Ligia* were made; but if it should prove that they emerge from the shingle to find sanctuary in the rocks, then evaporation will have served a useful purpose during the period of exposure.

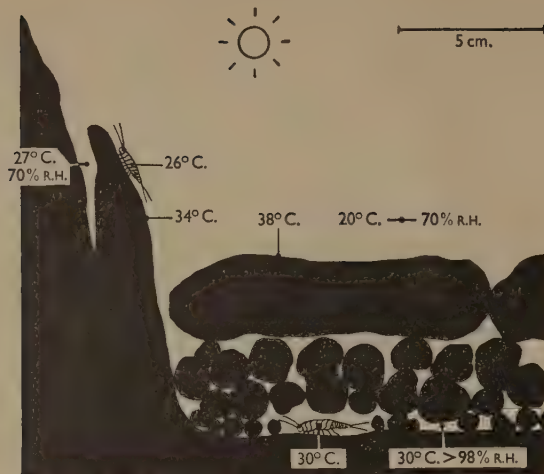


Fig. 5. Vertical section of base of red sandstone cliff and shingle inhabited by *Ligia* (diagrammatic). To show the microclimatic conditions and internal temperatures of the animals, at c. 14.00 G.M.T. in August 1951.

DISCUSSION

The temperature of a poikilothermal animal is that at which a balance is struck between gain and loss of heat. Heat is gained by metabolism and lost by evaporation; it may be either gained or lost by conduction, convection and radiation, depending upon the temperature of the surroundings. The interplay of these factors in determining temperature is complex and the subject has recently received attention from the theoretical point of view by Parry (1951), who shows that, for resting insects in direct sunlight, neither metabolism nor evaporation contributes significantly to the total heat balance. Since woodlice differ from insects in having a relatively permeable cuticle, we may expect to find evaporation of greater significance in this group.

We may now make a rough estimate of the heat exchange balance for *Ligia* during a more stable period of exposure, and it will be convenient to start with an earlier record which was briefly referred to elsewhere (Edney, 1952). The relevant temperatures were as follows: air 21°, living *Ligia* 28°, dry *Ligia* 34°, ground 35° C.

As regards *radiation*, Parry has shown (after Stagg, 1950) that the mean total radiation load (algebraic sum of input and output) upon a horizontal rectangular plate with a surface reflectivity of 50%, exposed to the sun within 2 hr. of noon, varies from 10 to 22 mW./cm.² (milliwatts/cm.²) according to its orientation. Assuming that the surface reflectivity of *Ligia* is also about 50%, its radiation load may be put at about 20 mW./cm.².

Conduction is an unknown factor, but is likely to be small, for *Ligia* is only in contact with the ground at the tips of its fourteen legs.

Parry found that the *convection* coefficient for a small disk in wind moving at 50 cm./sec. was 1.4 mW./cm.² for each ° C. by which the temperature of the disk differed from that of the air moving over it. *Ligia*, in the experiment being discussed, was 7° C. warmer than the surrounding air, which was moving gently (about 50 cm./sec.), so that it would lose heat at the rate of 9.8 mW./cm.².

No measurement of *evaporation* was made during this exposure, but it can be estimated from laboratory work, which shows that *Ligia* evaporates water at the rate of 11.5 mg./cm.²/hr. into dry air moving at 5 cm./sec. at 30° C. Now the rate of evaporation is roughly proportional to the difference between the vapour pressure of water in air saturated at the temperature of the evaporating surface (p_0) and the vapour pressure of water in the surrounding air (p_a) provided the temperatures do not differ widely (Ramsay, 1935*a*). In the laboratory measurements referred to, $(p_0 - p_a) \div 25$ mm. Hg (surface temperature of *Ligia* about 26° C., surrounding air dry), while in the field it was 16 mm. Hg. This means that the rate for *Ligia* in the field would be $16/25 \times 11.5$, or 7.4 mg./cm.²/hr. into slowly moving air. Now, at low wind speeds, the rate of evaporation from a free surface is approximately proportional to the square root of the velocity (refs. in Ramsay, 1935*a*); this was 5.0 cm./sec. in the laboratory, and some 50 cm./sec. in the field. At a conservative estimate we may therefore double the rate of evaporation, giving 14.8 mg./cm.²/hr., and this is equivalent to 9.9 mW./cm.².

In order to compare the effect of *metabolism* with other factors we need to know the metabolic rate in terms of surface area. This has been measured by Spencer (unpublished), who found that the O₂ consumption of *Ligia* at 25° C. is in the region of 0.02 ml./cm.²/hr., and this is equivalent to a heat production (at 4.775 cal./ml. O₂) of 0.11 mW./cm.². The effect of this rate of heat production in field exposures may be neglected. In laboratory experiments, where other factors are also low, metabolism may well affect the equilibrium temperature considerably. (An increase of 0.4° C. was recorded for *Ligia* in these conditions (p. 333).)

The heat exchange balance (in mW./cm.²) for *Ligia* in the conditions specified is now as follows:

$$20 \text{ (radiation)} + x \text{ (conduction)} = 9.8 \text{ (convection)} + 9.9 \text{ (evaporation)}.$$

The balance suggests that conduction is indeed small, and that in this particular exposure the parts played by evaporation and convection were roughly equal.

The acceptability of the above value for loss of heat by evaporation may be tested by calculating an expected rise in temperature if evaporation is omitted from the heat balance, and comparing this with the experimentally determined temperature of a dry *Ligia*.

If evaporation ceases, the temperature of the animal rises, and this increases the rate of loss of heat by convection and also decreases the net radiation load (since the animal will radiate more heat while accepting the same amount as before). For a dry animal then, the value of 9.9 mW. must be removed from the loss side of the

balance above, and, since a new balance is established, the value of 9.8 mW. for convection must increase. It will not increase by as much as the 9.9 mW. previously lost by evaporation, for the value for radiation on the input side of the balance decreases in the new situation: it will increase by 9.9 mW. less the drop in the radiation value. If c is the difference in convection loss and r the difference in net radiation load, $c = 9.9 - r$. (This equation can also be derived from equation (3) in the Appendix.)

It can also be shown (see Appendix) that in the conditions specified, and assuming an emissivity coefficient of 0.75 for *Ligia*, $r/c = 0.33$, and a solution of these simultaneous equations gives $c = 7.4$. In other words, there is an increase of 7.4 mW. in the convection loss value, and in order to bring about such an increase, where the convection coefficient is 1.4 mW./cm.²/° C., the temperature of the body must rise by 5.3° C. Calculation therefore leads us to expect that the difference in temperature between a living and a dry *Ligia* will be 5.3° C., and this brings the temperature of the dry animal to 33.3° C. The experimentally determined temperature was 34° C., so that the estimate of 9.9 mW. for evaporation is, if anything, too low; a value of 11.2 mW. for evaporation is necessary to give a calculated dry temperature of 34° C.

The above analysis refers to an exposure where the air temperature was lower than that of the living *Ligia*. In some exposures this was not so, particularly those carried out later in the day when radiation was lower. At 16.30 hr. total radiation is approximately half the maximum around noon (Stagg, 1950). In such exposures (e.g. Fig. 2), convection will act in the opposite direction, and heat will be gained by the animal from the air, though the rate of gain will not be high, for the air temperature is usually very near that of the animal. During this exposure, the rate of evaporation was estimated, by weighing the animal before and after. It was found to be 18 mg./cm.²/hr., which represents a heat loss of 12.0 mW./cm.². The heat exchange balance, in mW./cm.², during the period from 16.24 to 16.35 hr. shown in Fig. 2, might therefore work out somewhat as follows:

$$10 \text{ (radiation)} + 1 \text{ (conduction)} + 1 \text{ (convection)} = 12 \text{ (evaporation)}.$$

The figure for evaporation is high, but well within the powers of *Ligia*, for the highest rate of evaporation measured during exposure to the sun was 24 mg./cm.²/hr., and this rate was continued for 30 min., after which the animal was still alive. It represents a loss of about 25% of the total weight.

The curves for the other species of woodlice show that they were usually well above air temperature, which is due to the fact that they do not evaporate water so rapidly as *Ligia* does. There is little point in estimating heat exchange balance equations for each species: the principle is the same for all, and in any case it cannot be claimed that the equations given for *Ligia* are quantitatively accurate; their purpose is to show the relative importance of the factors involved.

There is a growing literature on the temperature of insects in the sun (e.g. Strelnikov, 1936), much of it on the Acridiidae, which has been reviewed by Uvarov (1948) (see also Wigglesworth, 1950). Unfortunately, in most of the work

on insects, there have been no dry specimens as controls, and it is therefore impossible to say to what extent the temperature is affected by evaporation. Buxton (1924) showed that on soil at 44° C. the Tenebrionid beetle *Adesmia* was at 39.5° C. if alive, and from 2 to 9° C. warmer if dead. The dead animal was not dry, however (Buxton, personal communication), so that these figures do not demonstrate the total effect of evaporation, but only the effect of greater evaporation from living than from dead beetles. We do not know the temperature of the air surrounding the beetle, or its velocity, so the effect of convection cannot be estimated.

In general, insects in sunlight seem to undergo very considerable and rapid increases in temperature, which is what would be expected from a small animal which evaporates water but slowly. The cockroach *Blatta*, used in the present experiments, behaves as might be expected. The rate of evaporation from this species has been measured by Gunn (1933, 1935), who showed that raising the temperature from 30 to 36° C. increases the rate, in still air, six times; and this, as Ramsay (1935*b*), Wigglesworth (1945) and others have shown, is due to a change in state of the wax in the epicuticle. Gunn's figures work out at about 2.0 mg./cm.²/hr. into still air at 36° C. Ramsay (1935*b*) has shown the great effect of wind velocity between 4 and 20 m./sec. on the rate of evaporation from the spiracles of *Periplaneta*, but there is no precise information about the effect of lower speeds upon evaporation from the cuticle above 30° C. Wind velocity during the exposure of *Blatta* which we are considering was the highest experienced during the whole series of experiments: it was estimated as 4 on the Beaufort scale, or about 500 cm./sec. (though it was gusty), so that evaporation may well have been something like 6.0 mg./cm.²/hr. If this figure is accepted, then the heat exchange balance for *Blatta*, at 15.24 hr. during the exposure figured, would be approximately as follows:

$$10 \text{ (radiation)} + 1 \text{ (conduction)} = 7 \text{ (convection)} + 4 \text{ (evaporation)}.$$

That evaporation, even in still air, can significantly reduce the temperature of the cockroach under laboratory conditions has been shown by Gunn & Notley (1936), who found that *B. orientalis* can survive exposure to 43° C. for an hour in dry air, but only 41° C. if the air is moist.

The ecological implications of solar radiation for arthropods are by no means clear. As regards insects, the effects of insolation which have so far been demonstrated are, speaking rather generally, beneficial, as Kennedy (1939) and others have shown for locusts, particularly as regards development and activity. The harmful effects of insolation causing a dangerously high body temperature, and the possible mitigating effect of evaporation in such conditions, have received little attention, although the evaporation effect has been amply demonstrated in the laboratory (Mellanby, 1932). Kragerus (1948), in the course of a far-reaching investigation of the ecology of strand insects in Finland, measured the body temperature, in the sun, of a number of Carabid beetles and obtained temperatures up to 26° C. higher than the surrounding air. He also investigated the effect of transpiration from these insects, but only in laboratory conditions, where radiation was negligible, and found temperature depressions of about 1.5–2.0° C. in unsaturated air.

Neches (1924) has claimed that, in the cockroach, temperature *regulation* by evaporation occurs, and extends the range of the species. But, as Gunn (1942) points out, this kind of regulation can hardly be compared with biological temperature regulation such as occurs in mammals, for it is a simple physical process, occurs in dead as well as living insects, and does not lead to the maintenance of a *constant* temperature. Furthermore, the temperatures at which evaporation is sufficiently rapid to produce significant cooling are far above those ever likely to be experienced by cockroaches in nature, if only because their orientation mechanisms lead to strong avoidance of such environments. It is still possible, of course, that harmful effects of insolation may be mitigated in other species of insects—sudden exposure to the sun's rays can, as we have seen, increase temperature very rapidly—but there is no information at all on the subject.

The land isopods present rather a different picture. Their integument is much more permeable to water than that of insects, so the effects of evaporation are more significant. There can be little doubt that by far the most important effect is to restrict them to relatively cool, moist microclimates; nevertheless, they do appear in direct sunlight, and the present work suggests that they may do so in two different sets of circumstances. Firstly, as in insects, when insolation is used to increase the body temperature above that of the surroundings, and thus to accelerate development; and secondly, when it is necessary to suffer insolation in order to avoid the combination of saturated air and high temperatures when this occurs in the normal habitat under stones, etc. (p. 341). In the second case, the ability to evaporate water rapidly is valuable.

Woodlice will also benefit from rapid evaporation if, as seems not unlikely, they are ever caught in exposed places by sudden sunshine, or forced to cross insulated areas in the search for favourable microclimates. There is little point in speculating further on the extent to which this occurs—observation in the field, coupled with precise measurement of the microclimates which obtain at the time, are necessary to settle the point.

As regards the orientation mechanisms which lead woodlice to exposure in insulated areas, little at present can be said. The general trend, as is well known from the work of Gunn (1937), Waloff (1941) and others, is for woodlice to show hygro-positive and photo-negative behaviour. However, behaviour may alter considerably in different circumstances. Cloudesley-Thompson (1952) has recently shown that the humidity reactions of *Oniscus* differ in intensity according to the light, and Henke (1930) showed that in *Armadillidium* some individuals exhibit positive phototaxis when the temperature rises. If a similar mechanism were present in *Ligia* we might have an explanation of the emergence of these animals from beneath hot stones into the sunlight. But further work in the laboratory and in the field is needed to test this suggestion.

SUMMARY

1. The effects of insolation and evaporation upon the internal temperature of the woodlice, *Ligia oceanica*, *Oniscus asellus*, *Porcellio scaber* and *Armadillidium vulgare*, and of the cockroach, *Blatta orientalis*, were investigated.

2. During insolation, the temperature of a thoroughly dry *Ligia* remains within 0.5°C . of that of a freshly killed specimen covered with beeswax. Dry specimens do not, therefore, differ significantly in shape from living ones, and are valid as controls when estimating the effect of evaporation from living animals.

3. Living, freshly killed, and dead, dry animals were exposed together for periods of 30 min. or more upon insolated rock in the natural habitat, upon slate, and upon wooden blocks which could be moved from shade to sun. Their temperatures, and those of the surrounding air and ground, were measured by fine thermocouples; humidity was measured by a small electric hygrometer, and air speed was estimated approximately.

4. In the sun, the temperature of a dead, dry animal is usually near that of the ground, and always higher than that of a living or a freshly killed specimen. The air temperature, 5 mm. above ground, may be either above or below that of *Ligia*, but it is always below that of the other species.

5. The amount of the temperature differences between ground, air, living and dry specimens of any one species varied from one exposure to another according to the intensity of radiation, the temperature and humidity of the air, and its velocity.

6. When all species were exposed together, they showed, during a stable period, approximately the following temperature depressions (from ground temperature): *Ligia*, 8.0°C .; *Oniscus*, $4-5^{\circ}\text{C}$.; *Porcellio*, $2-3^{\circ}\text{C}$.; *Armadillidium*, 4°C . The species stand in the same order as they do in respect of rates of evaporation, except for *Armadillidium*, and it is suggested that this species absorbs less radiant energy per unit area by reason of its shiny surface and arched dorsum. *Blatta* shows a temperature depression between those of *Porcellio* and *Oniscus*.

7. Measurements made in the natural habitats of *Porcellio* and *Ligia* suggest that these animals are exposed to the sun, (a) when the resulting increase in body temperature brings it nearer the optimum for development, and (b) when insolation is incurred during locomotion from hot, saturated microclimates (e.g. under insolated stones) to more favourable ones. During exposures of type (b), rapid evaporation is advantageous, as a means of avoiding dangerously high temperatures.

8. Radiation, conduction, convection, evaporation and metabolism are considered with regard to their effect upon equilibrium temperature. Conduction and metabolism are shown to be negligible during exposure to direct sunlight. Heat exchange balances, applying to *Ligia* and *Blatta* during particular exposures, and involving radiation, convection and evaporation, are estimated in terms of milliwatts/cm.². If evaporation is eliminated, the effect upon the two remaining terms, and the temperature at which a new balance is reached, can be calculated approximately. The temperature of a dry *Ligia*, so calculated, corresponds with the experimentally measured temperature.

9. The results are compared with what is known of the effects of insolation upon the temperature of insects. Evaporation is likely to play a more important part in determining the temperature of woodlice than of arthropods with an impermeable cuticle.

I am grateful to Prof. Lancelot Hogben, F.R.S., for valuable discussions on the general problems involved in this work.

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APPENDIX

Calculation of the effects of evaporation and convection upon temperature

For the purpose of the following calculations, the effects of metabolism and conduction, both of which are known to be relatively small, are neglected.

(a) To find the difference in temperature between a dry body and one which loses water by evaporation.

Assume two bodies with identical physical properties, except that one may lose heat by evaporation. Let the temperature ($^{\circ}$ Abs.) of the evaporating body be T and that of the dry body be T' .

The net energy input to either body, due to radiation, is assumed to be of the form $(A - 4.28 \times 10^{-9} T^4)$ mW./cm.². This expression assumes that external conditions remain constant, thereby giving rise to a constant energy input (A), and that the mean emissivity coefficient for the body within the temperature range concerned is 0.75.*

If the energy loss due to evaporation from one body is E mW./cm.², then the energy balance equations for the two bodies will be

$$A - 4.28 \times 10^{-9} T^4 = E + C(T - T_a), \quad (1)$$

$$A - 4.28 \times 10^{-9} T'^4 = C(T' - T_a), \quad (2)$$

the additional term representing the convection energy loss, C being dependent upon wind speed, (T_a = air temperature.)

Subtracting equation (2) from equation (1),

$$4.28 \times 10^{-9} (T'^4 - T^4) = E - C(T' - T). \quad (3)$$

Let $T' - T = t$. If t is much smaller than T , the equation simplifies to

$$4.28 \times 10^{-9} \times 4 T^3 t = E - Ct,$$

that is

$$t = \frac{E}{1.71 \times 10^{-8} T^3 + C}.$$

(b) To find the extent to which radiation and convection respectively affect the difference in temperature, let the difference in net radiation input be r , and in convection loss be c .

Then

$$r = 4.28 \times 10^{-9} (T'^4 - T^4),$$

$$c = C(T' - T).$$

* This figure may well be too low so far as *Ligia* is concerned, for the animal will radiate in the infra-red; but *ceteris paribus* a higher emissivity coefficient implies a larger value for evaporation in the heat exchange balance (see p. 343). Since the present work is partly concerned to show the importance of evaporation in this respect, it is advisable to err, if at all, in the opposite sense.

As before, let $t = T' - T$,

$$\frac{r}{c} = \frac{4.28 \times 10^{-9} \times 4T^3 t}{Ct} = \frac{1.71 \times 10^{-8} T^3}{C}.$$

If $C = 1.4$,* and $T = 300$,

$$\frac{r}{c} = 0.33.$$

The use of the first term of the binomial expansion in each of the above solutions involves an error of about 4% for a value of $t = 7^\circ \text{C}$.

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* For a $1 \times \frac{3}{8}$ in. brass disk in a wind speed of 50 cm./sec. (Parry, 1951).

THE OCCURRENCE OF BLOOD GROUPS IN CATS

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(With Plate 10)

INTRODUCTION

The investigations of Ingebrigsten (1912) and of Ottenburg & Thalhimer (1915) indicated that cats could not be grouped on the basis of the isoagglutination reactions of their blood, but the experimental methods used by these workers are open to certain criticisms which make it necessary to re-examine some of their conclusions.

However, using techniques based on those suggested for use in investigations on human blood groups (Schiff & Boyd, 1942; M.R.C. memo no. 9, 1943; Wiener, 1943), it has been shown (Holmes, 1950) that there are at least two blood groups in cats: group I, those having an isoagglutinin in the cells, forming about 97% of the population; group II, the remainder, lacking the isoagglutinin but having the homologous isoagglutinin in the serum.

The reaction between cats' cells and sera obtained from group II animals has been used to classify members of the cat population with respect to their isoagglutinin-isoagglutinin make-up. During such a routine grouping of a number of animals it was noticed that the red blood cells of one cat gave a delayed and distinctly weaker agglutination reaction with antiserum from group II animals than did the blood of other individuals which had been previously tested and classified as group I. A more detailed investigation of the properties of this weakly reacting blood demonstrated conclusively that it could not be classified as either group I or group II, but that a further grouping was present in the cat population. Animals belonging to this third group have blood possessing both an isoagglutinin and an isoagglutinin.

MATERIALS AND METHODS

Adult animals, constituting a random sample of local cats, have been used throughout.

Specimens of serum were obtained by bleeding into a sterile tube from a small incision on the undersurface of the cat's ear near the edge. The blood was allowed to coagulate at room temperature (16–20° C.) for 1 hr. and at ice-box temperature (3° C.) for 1–2 hr., after which the retracted clot was removed and the serum centrifuged. The clear serum was pipetted into small, sterile glass bottles and inactivated at 56° C. for 30 min. to destroy complement and thus prevent haemolysis

* Part of this work was performed during the tenure of a Medical Research Council Studentship.

during agglutination tests. Preservation was effected by storage at -10°C . Kept in this way serum retains its strength and specificity indefinitely.

Approximately $2\frac{1}{2}\%$ red-cell suspensions were prepared by adding one drop of blood to 1 ml. of a citrate-isotonic saline solution. Owing to the gradual deterioration of the blood suspensions only those less than 6 hr. old were used for a test.

Before the discovery of the third cat group, a quick slide method had been used for grouping unknown bloods, but since that time a tube method has been used in every case. The former consisted of agitating a slide bearing one drop of a solution of a strong antiserum (1 vol. of serum to 1 vol. of isotonic saline) tinged faint pink with whole blood; agglutination if it were going to occur showed macroscopically in from 1 to 2 min. In the tube method, performed in duplicate, one drop of a $2\frac{1}{2}\%$ red-cell suspension of the unknown blood was mixed with one drop of diluted antiserum in a small test-tube and, after standing at room temperature for 15 min., was centrifuged at 2500 r.p.m. for 2 min. After resuspending the sediment by flicking the tube gently with the finger readings were made both macroscopically and microscopically. Adequate controls were included with every test, namely, saline plus red-cell suspensions of the three known groups; and antiserum plus red cells having no isoagglutinin. Bloods showing no agglutination with the grouping serum and also those giving doubtful results were re-investigated by testing their sera against red cells of known group in addition to testing their red-cell suspensions with several samples of antiserum from different animals. A further check in cases of putative group II blood consisted of absorbing a serum sample with cells of the third group and testing the absorbed fluid against known cells. If the suspected blood were indeed group II, the absorbed serum still reacted with group I cells; an example of this is illustrated in Pl. 10, fig. 2.

Two agglutinin solutions have been used in routine grouping of batches of cats: (1) serum from the third group diluted with an equal volume of isotonic saline; and (2) a pure agglutinin fluid prepared as follows: ten drops of washed, packed group III red cells were mixed with 3 ml. of strong group II serum and occasionally agitated at room temperature for 1 hr. The agglutinated cells were separated by centrifuging and washed several times to remove unwanted group II sera. One and a half ml. isotonic saline was added to resuspend the sediment and the mixture heated slowly to 56°C ., at which temperature it was maintained for 5 min. At 56°C . the process of agglutination is reversed, the isoagglutinin being released from the agglutinated red cells into the saline. This suspension was centrifuged in tubes surrounded with water at 56°C . for 2 min., the supernatant removed and centrifuged once again for 5-10 min. The final supernatant contained the pure isoagglutinin ready for use.

Full absorption of an isoagglutinin from an antiserum was obtained by adding 0.5 ml. of washed, packed red cells to 1 ml. of serum and centrifuging after standing for half an hour at room temperature. This process was repeated with half the volume of packed red cells used in the previous absorption, until testing the absorbed serum with the absorbing red cells gave no agglutination. Usually three absorptions were necessary to remove an isoagglutinin completely.

Titration were performed in tubes, the antisera being diluted with saline to prepare serial twofold dilutions and a 1% red-cell suspension, prepared each time from the same group I animal, used as a standard test cell. After standing for 2 hr. the sediment was resuspended and examined for agglutination. Titres were expressed as the reciprocal of the final dilution of antiserum in the last tube showing definite microscopic agglutination.

The presence of haemolysis has been determined by means of the tube method as described but using fresh, unheated serum and incubating at 37° C. for 2 hr. Any haemolysis that occurred was easily detected by comparing the red stained supernatant with the unaffected controls. It was usual to include test cells plus inactivated serum as one of the controls.

Cold agglutinins were investigated by mixing serum separated at 37° C. with an equal volume of a 2½% suspension of washed red cells and leaving overnight at 3° C. Any sample exhibiting agglutination was incubated at 37° C. for 2 hr. during which agglutination should be reversed, for cold agglutinins are generally inactive at 37° C.

RESULTS

The results of cross-agglutinating the red cells and sera of the three cat groups are shown in Pl. 10, fig. 1. This figure was prepared by performing the tests in tubes as described and allowing one drop of the resuspended sediment to fall on the appropriate square of a cross-hatched filter-paper. After drying in the air the filter-paper retains the red-cell stains for years, the difference between agglutinated and non-agglutinated samples remaining clear and distinct. The system of nomenclature shown also in Pl. 10, fig. 1, can only be considered tentative and is used not to define clearly the agglutinin constitution of the three groups but only as a working classification in the light of the known facts.

The reactions of cat group *O* serum with cells from groups *EF* and *F* can be most easily explained by assuming that such a serum contains at least two isoagglutinins capable of agglutinating cells from one or both of the other groups. This supposition is supported by the results of the absorption experiments illustrated in Pl. 10, fig. 2. Absorption of group *O* sera with group *F* cells removed only one isoagglutinin, for, on testing the absorbed serum with group *EF* cells agglutination still occurs indicating that a second isoagglutinin still remains. Thus it would appear that group *O* sera contain anti-*E* and anti-*F* isoagglutinins, both of which can be removed by group *EF* cells. On this basis group *EF* has no serum isoagglutinin content while the antibody present in group *F* blood is limited to anti-*E*.

Anti-*F* isoagglutinin only occurs in group *O* sera, and here it is always present together with anti-*E*. However, by eluting the antibody from agglutinated group *F* cells a pure agglutinin fluid containing anti-*F* can be obtained. The results of testing this antibody with cells of the cat groups is shown in Pl. 10, fig. 3.

Properties of isoantibodies

Titration of sera obtained at intervals from groups *O* and *F* cats have been performed over periods ranging from several months to 2 years. In not a single case

has the antibody strength varied markedly from its initial value. The antibodies in groups *O* and *F* sera have also retained the specificity of their reaction with cells of all groups, as demonstrated by occasional testing.

With respect to thermal amplitude both antibodies are most active at approximately 18° C., the activity falling off gradually with increase in temperature until at about 56° C. agglutination is inhibited but without destruction of the antibody. At around 3° C. antibody activity is significantly lower than at room temperature.

Haemolysins. Haemolysis regularly occurs, especially at 37° C., with fresh sera from groups *O* and *F*. However, the haemolysins responsible are only specific for *EF* cells, red cells from groups *F* and *O* under similar conditions remaining unaffected. Group *EF* sera do not appear to possess haemolysins against any cat red cells.

Cold agglutinins. Antibodies active at about 3° C. occur occasionally. They are infrequently present in blood from group *O* and from group *EF* animals and are non-specific in character, almost invariably acting as autoagglutinins of their own red cells. In all cases the cold agglutinins react weakly, never having a titre greater than 4-8 at 3° C. Blood coagulated at 3° C. invariably yielded serum which was uncontaminated by non-specific cold agglutinins, these having been absorbed by the red cells in the clot.

Recently, a weak agglutinin most active in the cold has been found in the serum of a group *EF* animal; it reacts with cells of groups *O* and *F* but not with group *EF* blood. This antibody, which reacts very weakly at room temperature and not at all at 37° C., is under investigation.

Frequency of occurrence of cat groups

Using anti-*E* and anti-*F* solutions as testing media, the groups of 103 cats have the following incidence:

Groups ...	<i>EF</i>	<i>O</i>	<i>F</i>
Absolute numbers	98	4	1
Percentage	95.15	3.88	0.97

The values for the occurrence of groups *EF* and *O* in the cat population are very similar to those reported previous to the appearance of group *F* and using group *O* serum as the single testing fluid (Holmes, 1950). In the latter case in a population of 477 cats, 97% were group *EF* and 3% group *O*.

DISCUSSION

The technique used by Ingebrigsten and by Ottenburg & Thalheimer in their cross-agglutination experiments was based on that described by Epstein & Ottenburg (1909). In the experiments of Ingebrigsten this consisted of mixing 2 vol. of serum with 1 vol. of a 5% red-cell suspension in a capillary pipette and investigating macroscopically after 2 hr. at 38° C. and again after 24 hr. in the ice box. In this author's own words, 'The reaction was recorded as positive only when...the agglutination was plainly seen on the first as well as on the second examination. In two or three cases the agglutination was very strong and occurred some minutes

after the mixture had been made; but usually it was not marked until the next day, i.e., after 24 hours in the ice chest.' This statement suggests that Ingebrigsten may have been overlooking certain examples of true isoagglutination by not including in his protocols as positives those cases where agglutination was marked only after 24 hr. at 3° C. However, owing to the fact that Ingebrigsten gives no indication of the conditions used during the preparation of the sera, it is impossible to infer whether those examples of agglutination only noticeable after 24 hr., were due to the activity of non-specific cold agglutinins or to true isoagglutinins too weak in their reaction at 38° C. to be definitely observed macroscopically. These considerations alone make questionable whether Ingebrigsten was recording the correct results of certain of his tests and thus his conclusions must remain open to doubt.

Beyond indicating that readings were made generally after 2 hr. at room temperature Ottenburg & Thalhimers description of the investigations into normal cat isoagglutinins omits any reference to the proportions of serum and red-cell suspension used for a test; nor do they state the concentration of erythrocyte suspensions prepared. However, in the section of their paper devoted to the appearance of haemolysins after transfusion, mention is made to 5% red-cell suspensions used in testing for haemolysins, and in an earlier paper concerned with the results of investigations into grouped isoagglutinins in rabbits and steers (Ottenburg & Friedman, 1911) the tests are described as comprising 3 vol. of serum plus 1 vol. of a 3% or 5% red-cell suspension. Thus, from these statements, it is highly probable that similar techniques were in use for the cat isoagglutinin experiments, namely, 2 vol. or 3 vol. of serum mixed with 1 vol. of an approximately 5% suspension of cells. Assuming that this is the case, let us consider some of Ottenburg & Thalhimers findings. First, they reached the conclusion that agglutinins when present were not constant in power or specificity but varied considerably from time to time. This has not been found to be the case in the present investigation. Secondly, their statement that, 'isohaemolysins do not occur among normal cats', is also false, the presence of isohaemolysins having been clearly demonstrated. Thirdly, they find that autoagglutination sometimes occurred; moreover, the autoagglutinative sera were usually those which affected the red cells of a large number of other animals. In their protocol illustrating the results of cross-agglutination of serum and cells from fifteen animals two examples of thirty-eight recorded positives are cases of autoagglutination. Cold agglutinins have been conclusively demonstrated in the present study, and it is conceivable that the autoagglutinations observed by Ottenburg & Thalhimers were, in fact, examples of cold agglutinins still reacting weakly at room temperature. Alternatively, they could have been non-specific false reactions simulating and indistinguishable from true isoagglutination. Now, because these workers listed examples of autoagglutination among their positive reactions it is necessary to inquire, how many more out of the remaining thirty-six recorded positives were in fact cases of non-specific agglutination. In view of this it is possible that had only true isoagglutination been considered the presence of distinct groups might have been revealed. Because of this possible source of error their results cannot be accepted without reservation.

The methods used by Ingebrigsten (and most probably by Ottenburg & Thalhimer) have not given clear-cut results in this laboratory. Ingebrigsten especially did not perform his experiments under optimum conditions for the antigen-antibody reaction. Furthermore, false reactions are more liable to appear when using this technique. The methods used in the present investigations are not subject to these errors. They are based on methods which have been used so successfully in human serology and, with cat blood, they have given consistently unequivocal results in which the possibility of false reactions remaining unrecognized has been reduced to a minimum.

The results shown in Pl. 10, figs. 1-3, bear a striking resemblance to those obtainable using human sera and red cells of groups *O*, *AB* and *A* (or *B*) for the tests. Though this similarity provided a guide in choosing the tentative nomenclature of the groups no more than an analogy is claimed to exist between the reactions of the cells and sera of cats on the one hand, and the cells and sera of humans on the other. Assuming that the nomenclature shown in Pl. 10, fig. 1, does represent the actual agglutinin content of the red cells, and bearing in mind the fact that there are four groups in man, it seems feasible that a fourth grouping exists in the cat population. If this is so, then this fourth group ought to have an agglutigen *E* in the red cells with agglutinin anti-*F* in the sera. However, no cat group having this composition has yet been identified.

If we assume that the inheritance of the cat groups is determined by a genetical system similar to the human *ABO* model, it is possible to calculate the theoretical frequency of occurrence of group *E* in the population. Let *x*, *y* and *z* be the frequencies of the genes, *E*, *F* and *O* respectively, then in a homogeneous population $x + y + z = 1$.

Phenotype	Genotype	Frequency
\bar{O}	<i>OO</i>	z^2
\bar{E}	<i>EE, EO</i>	$x^2 + 2xz$
\bar{F}	<i>FF, FO</i>	$y^2 + 2yz$
\bar{EF}	<i>EF</i>	$2xy$

$$z = \sqrt{\bar{O}},$$

$$\bar{O} + \bar{F} = y^2 + 2yz + z^2 = (y + z)^2,$$

$$y + z = \sqrt{(\bar{O} + \bar{F})},$$

or,

$$x = 1 - \sqrt{(\bar{O} + \bar{F})}.$$

From the observed values of groups *O* and *F*

$$x = 1 - \sqrt{(0.0388 + 0.0097)} = 0.7798$$

and

$$z = \sqrt{0.0388} = 0.197.$$

Now

$$\begin{aligned} \bar{E} &= x^2 + 2xz \\ &= 0.7798^2 + 2 \times 0.7798 \times 0.197. \\ &= 0.915 \text{ or } 91.5\%. \end{aligned}$$

It is obvious that the theoretical value obtained for group *E* bears no relation to the observed value. In the population considered group *E* is completely lacking, whereas if the three-gene theory is tenable this group should characterize the blood of more than 91% of animals tested. The enormous discrepancy between the observed and calculated frequencies of group *E* suggest that the theory of triple allelomorphic genes as applied to cats does not adequately account for the known facts. Further information on this point could be obtained by breeding experiments, for, on the three allelomorphic gene theory, $EF \times O$ matings should give equal numbers of *E* and *F* offspring and no *EF* or *O*. Breeding experiments such as these are in progress but no definite conclusions are available from them at the present time.

A possible explanation of the cat data could be that a system similar to the A_1A_2O of humans is present. On this basis, E_1 would be equivalent to *EF* and E_2 would represent *F*. Group *O* serum would contain anti-*E* and anti- E_1 while group E_2 serum would have anti- E_1 only. If such a theory were indeed valid, then the group *E* postulated on the human *ABO* model would never be found. Some evidence for the E_1E_2O hypothesis is provided by the existence of an antibody present in group *EF* serum and active against groups *O* and *F* cells. It is possible that this cold agglutinin is the analogue of human anti-*O* or anti-*H* found sometimes in groups A_1B and A_1 blood.

An alternative hypothesis is that a system similar to the *Rh* system in man is operative. Here there would be two closely linked loci, one occupied by either *E* or *e* and the other by *F* or *f*. On such a theory, the absence of group *E* could be explained by the fact that the chromosome *Ef* might not exist or be so rare that its detection would be impossible. This is a similar example to the very rare chromosomes *CDE* and *CdE* of the human *Rh* system.

It is impossible to determine which of the two alternative genetical systems is actually in operation from the present knowledge. Breeding experiments could not be used as a means of differentiation for they could only help to eliminate the triple allelomorphic theory. However, a decision may be reached serologically for the demonstration of separate antibodies against *E* and *e*, and *F* and *f* would support the *Rh* model.

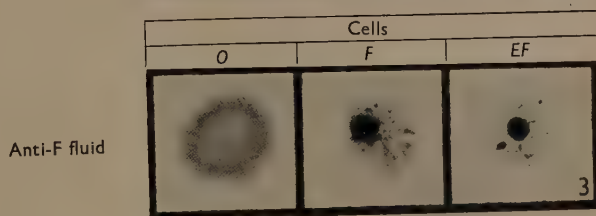
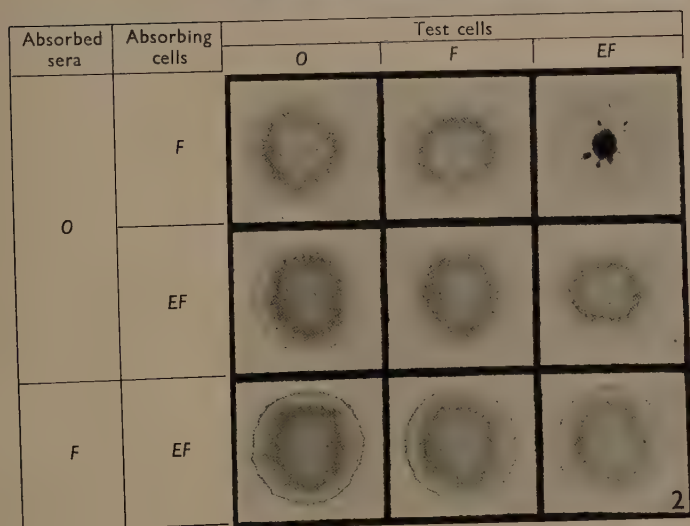
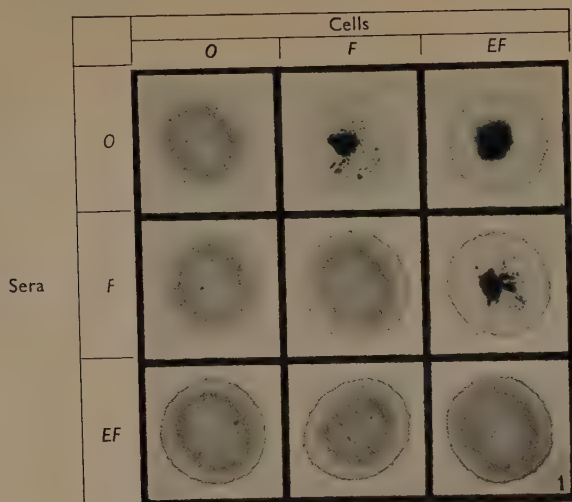
SUMMARY

Methods are described which have been used to investigate isoagglutination reactions of the blood of cats. Evidence is presented which indicates that cats possess at least three groups. The methods and results of earlier workers which indicated that grouped isoagglutinins did not occur in cats have been criticized.

A genetical system for the cat groups similar to the human *ABO* system is shown to be untenable.

Two other possible systems similar to the A_1A_2O , and to the *Rh* models of humans are suggested and briefly discussed.

I wish to express my appreciation to Dr A. E. Mourant, Director of the Medical Research Council Blood Group Reference Laboratory, for much helpful advice and criticism.



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EXPLANATION OF PLATE

- Fig. 1. The results of cross-agglutinating sera and cells from the three cat groups. Black stains represent agglutination; greyish patches, non-agglutination.
- Fig. 2. The results of testing absorbed cat sera with red cells from the three groups.
- Fig. 3. The effect of anti-F fluid prepared from group O sera upon red cells from the three blood groups.

ACTIVE TRANSPORT OF POTASSIUM BY THE MALPIGHIAN TUBULES OF INSECTS

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INTRODUCTION

The study of excretion in Malpighian tubules by the direct method of collecting the fluid from them and analyzing it has had to await the development of methods capable of handling the relatively small volumes obtainable. A beginning has been made with the blood-sucking bug *Rhodnius* (Ramsay, 1952) and with the larva of the mosquito *Aedes* (Ramsay, 1950, 1951, 1953).

In *Rhodnius* the haemolymph usually contains very much more sodium than potassium. In the fluid collected from the tubules (which will be called 'urine' in this paper), as compared with the haemolymph, the concentration of potassium is greater and that of sodium is less. Since the blood on which the insect feeds contains more potassium and less sodium than the haemolymph and since the excretory mechanism is presumably adapted to maintain the normal ionic ratio in the haemolymph, this greater concentration of potassium in the urine is entirely in accordance with expectation.

The picture in *Aedes* is somewhat different. As in *Rhodnius* the haemolymph contains very much more sodium than potassium and the urine always contains more potassium and less sodium than the haemolymph. Both these ions are reabsorbed into the haemolymph from the urine in the rectum. Although the larva of *Aedes aegypti* is a fresh-water animal, it can survive in solutions of pure NaCl or KCl, of concentration approaching 1%. Under these conditions the cation present in the external medium enters the haemolymph through the anal gills and leaves by the Malpighian tubules; reabsorption from the rectum is not complete, so that the cation entering the body is eliminated via the anus. It is surprising, however, to find that when the animal is kept in a solution of pure NaCl, a condition in which there is a substantial flux of sodium through the body, the concentration of potassium in the urine is always greater than in the haemolymph and the concentration of sodium is always less. Further, when the animal is kept in a solution of pure KCl, under which condition it may be supposed that the excretory mechanism is actively eliminating potassium, there is still considerable reabsorption of potassium from the rectum. It follows that under all conditions there is a circulation of potassium within the body, from the haemolymph into the tubule and from the urine back into the haemolymph via the rectum or the mid-gut.

In the case of *Rhodnius* it is reasonable to interpret the high concentration of potassium in the urine as adaptive to the animal's immediate excretory problem;

but in *Aedes* we cannot regard it as adaptive since it persists under conditions in which the intake of potassium is zero. If it is not adaptive is it simply fortuitous or is it in some way connected with the fundamental processes of urine formation in Malpighian tubules?

Clearly the next step must be to extend these observations to other insects and determine the concentrations of sodium and potassium in haemolymph and urine.

A further question will then be posed: are the differences in concentration the result of active transport or can they be accounted for by passive diffusion? When the movements of electrolytes are under consideration it is not sufficient to know the differences in concentration; it is also necessary to know the difference in electrical potential across the wall of the tubules (see Ussing, 1949). The scope of the investigation has therefore been widened to include measurements of potential difference (p.d.) as well as analysis for sodium and potassium.

In approaching the general problem two considerations must be borne in mind. First, the structure of the Malpighian tubules and their anatomical relations with other organs of the body present a great range of variation; it is reasonable to suppose that this anatomical diversification is not unreflected in their physiological activities. One particularly striking disposition is known as 'cryptonephridism' (Poll, 1934). The distal (alternatively, upper or blindly-ending) portions of the tubules are closely applied to the wall of the rectum and enclosed with it in a 'perinephric' membrane which separates the tubules from the haemolymph. Cryptonephridism is widespread in Coleoptera, and is also found among the larvae of Lepidoptera where it seems to have been independently evolved (Poll, 1938). In relation to a possible circulation of potassium this arrangement is suggestive. Secondly, there is wide variation in the normal sodium/potassium ratio of the haemolymph from one species of insect to another. Boné (1944) has shown that these variations are correlated with feeding habits, carnivorous insects having high ratios, of the order of 20, and herbivorous insects having low ratios, of the order of 0.5 or less. It may be that these variations are directly related to the sodium/potassium ratio of the food, but it is also possible that the normal ratios are maintained by the activity of the excretory system.

A survey of insect excretion, such as has been contemplated above, must clearly be planned to bring these known anatomical and physiological relations within its scope. On the other hand, choice of material is limited by technical considerations and by availability. In the work which this paper describes eight species of insect have been investigated. This is a modest figure; but, as is brought out in the Discussion, a substantial range of conditions has been covered.

MATERIAL AND METHODS

The following insects have been used in this investigation:

- Locusta migratoria migratorioides* R. & L., adult (Orthoptera, Acridiidae).
- Dixippus morosus* (al. *Carausius morosus*), adult (Orthoptera, Phasmidae).
- Pieris brassicae* L., larva (Lepidoptera, Pieridae).

Dytiscus marginalis L., adult (Coleoptera, Dytiscidae).

Tenebrio molitor L., larva (Coleoptera, Tenebrionidae).

Tabanid larva (species not identified) (Diptera, Tabanidae).

Aedes aegypti L., larva (Diptera, Culicidae).

Rhodnius prolixus Stål., adult (Hemiptera, Reduviidae).

A brief description of the excretory system in each of these insects is given in Appendix I, with notes on the operations carried out.

In the previous work on *Aedes* the urine was collected by means of a cannula inserted through the anus into the intestine as described in an earlier paper (Ramsay, 1951). The urine of *Rhodnius* was collected directly from the tubules by piercing the wall with a pipette, but the operative technique used at that time was difficult and unsatisfactory. Means have now been devised which make the operation relatively easy, and these are described in Appendix II.

The analysis by flame photometry is described in detail by Ramsay, Brown & Falloon (1953). It is necessary, however, to add a word about the accuracy of the method. The full potential accuracy is only realized when it is possible to anticipate the order of concentration which is to be measured, so that the apparatus can be previously calibrated over a relatively narrow range. When the order of concentration cannot be anticipated, as in the present work, it is necessary to adjust the apparatus so that it will deal with the extreme limits of concentration which it is reasonable to expect, and this entails some loss of accuracy. This loss of accuracy is difficult to estimate exactly. The figures in Table 1 are qualified by 'estimated error', and this represents the error which in the writer's opinion may effect the analysis in question. Greater accuracy could of course have been achieved by repeating the experiments after the order of concentration had been established; but this would have taken time and the results as they stand are deemed adequate to support the conclusions which are to be drawn from this survey.

The operative technique used for the collection of urine is readily adaptable to the measurement of p.d. across the wall of the tubule. A pipette electrode is inserted into the tubule and an indifferent electrode is dipped into the haemolymph (see also Appendix II).

RESULTS

The concentrations of sodium and potassium in haemolymph and urine are given in Table 1, from which it can be seen that in every case the concentration of potassium in the urine is substantially greater than in the haemolymph. The difference in concentration is more marked in the aquatic insects (*Dytiscus*, *Aedes*, Tabanid) and in *Rhodnius* than in the other terrestrial insects. The concentration of sodium in the urine is generally less than in the haemolymph, but the difference is not so marked as in the case of potassium.

The results of the p.d. measurements are summarized in Table 2, but before they are further considered it is necessary to describe certain observations which, although merely incidental to the measurements, reflect upon them. The normal procedure was (1) to measure the asymmetry potential with both electrodes in the haemolymph, (2) to penetrate the tubule and to thrust the pipette in until the p.d.

reached a constant value, (3) to withdraw the pipette and measure the asymmetry potential again. As an alternative to withdrawing the pipette it was sometimes convenient to thrust it out through the wall of the tubule. While this second penetration, from within outwards, was being made it was often possible to measure a stable

Table 1. *Analysis of haemolymph and urine*

(Concentrations in m.equiv./l.)

	Haemolymph, C_2		Urine, C_1		$\frac{C_1}{C_2}$ (Na)	$\frac{C_1}{C_2}$ (K)
	Na	K	Na	K		
<i>Locusta</i> (1)	75 \pm 10	20 \pm 3	31 \pm 5	167 \pm 10	0.41	8.3
(2)	74 \pm 10	15 \pm 3	66 \pm 5	79 \pm 10	0.89	5.3
			41 \pm 5	146 \pm 10	0.55	9.7
(3)	102 \pm 10	22 \pm 3	128 \pm 10	73 \pm 10	1.25	3.3
			95 \pm 10	95 \pm 10	0.93	4.3
					Av. 0.81	6.2
<i>Dixippus</i> (1)	14 \pm 3	16 \pm 3	13 \pm 3	160 \pm 10	0.93	10.0
			6 \pm 3	186 \pm 10	0.43	11.6
			3 \pm 3	145 \pm 10	0.21	9.1
			7 \pm 3	118 \pm 10	0.50	7.4
(2)	14 \pm 3	17 \pm 3	4 \pm 3	144 \pm 10	0.29	8.5
			1 \pm 3	148 \pm 10	0.07	8.7
			6 \pm 3	125 \pm 10	0.43	7.3
			5 \pm 3	128 \pm 10	0.36	7.5
					Av. 0.40	8.8
<i>Pieris</i> (1)	9 \pm 3	30 \pm 3	8 \pm 3	165 \pm 10	0.89	5.5
(2)	5 \pm 3	27 \pm 3	3 \pm 3	144 \pm 10	0.60	5.3
			3 \pm 3	191 \pm 10	0.60	7.1
					Av. 0.70	6.0
<i>Tenebrio</i> (1)	77 \pm 5	32 \pm 5	14 \pm 3	283 \pm 10	0.18	8.8
(2)	54 \pm 5	53 \pm 5	17 \pm 3	217 \pm 10	0.31	4.1
					Av. 0.24	6.4
<i>Dytiscus</i> (1)	140 \pm 15	5 \pm 2	44 \pm 5	156 \pm 10	0.31	31.1
Tabanid (1)	151 \pm 15	5 \pm 2	25 \pm 5	160 \pm 10	0.17	3.20
<i>Rhodnius</i> (1)	158 \pm 15	6 \pm 2	108 \pm 10	79 \pm 10	0.68	13.2
			93 \pm 10	119 \pm 10	0.59	10.8
(2)	158 \pm 15	4 \pm 2	165 \pm 10	62 \pm 10	1.04	15.5
			113 \pm 10	89 \pm 10	0.71	22.2
					Av. 0.75	17.7
<i>Aedes</i> *						
Dist. water adapted	87	3	24	88	0.28	29.3
NaCl adapted	113	3	71	90	0.63	30.0
KCl adapted	87	6	23	138	0.26	21.0

* Average values (from Ramsay, 1953).

p.d., obviously differing from that measured with the pipette in the lumen. It seems likely that in these cases the pipette had entered the cells of the tubule wall and that the p.d. thus measured was developed across the membranes separating the cytoplasm from the haemolymph. Some examples are given in Table 3 from the insects in which this effect was most clearly seen, namely *Pieris*, *Tenebrio* and *Dytiscus*. In these insects the p.d. lumen/haemolymph is positive and the p.d. cells/haemolymph is negative. In four cases, however, a negative p.d. was measured

when the pipette was judged to be fairly in the lumen, although owing to the opacity of the tubule it was impossible to be quite certain of this. These four measurements are included in Table 2, in brackets, but they have been disregarded in calculating the average figures.

Table 2. *Measurements of p.d. across wall of tubule*

(The figures are millivolts. A positive sign indicates that the interior of the tubule was positive with respect to the haemolymph.)

Insect		Measurements	Av.
<i>Locusta</i>	(1)	+1, -16, -13, -11, -12, -8, -15	-16
	(2)	-19, -17, -21, -25, -22, -21, -18, -12	
<i>Dixippus</i>	(1)	+8, +16, +20, +31	+21
	(2)	+18, +9, +22, +30	
	(3)	+29, +16, +19, +34, +31, +29, +15, +21	
<i>Pieris</i>	(1)	+10, +7, +28, +26, +28	+28
	(2)	+22, (-35), +36, +42	
	(3)	+31, +22, +37, +47	
<i>Tenebrio</i>	(1)	+52, +27, (-10), +58	+45
	(2)	+30	
	(3)	+35, +54, +62	
<i>Dytiscus</i>	(1)	+23, +23, (-43), +27, +17, +21	+22
	(2)	+18, +28, +23, (-47), +23	
<i>Rhodnius</i>	(1)	-47, -45, -20, -32, -28, -57, -32	-35
	(2)	-25, -34, -39, -29, -29	
<i>Aedes</i>			
Dist. water adapted	(1)	+24, +30, +24	+21
	(2)	+8, +29, +18, +15	
NaCl adapted	(1)	+10, +22	+13
	(2)	+7, +10, +16, +13	
KCl adapted	(2)	+12, +15, +23	+16
	(2)	+15, +13, +16	

Table 3

(The figures after the insects serve to identify them with the individuals correspondingly numbered in Table 2. The figures for p.d. are in millivolts. For further explanation see text.)

Insect	P.d. lumen/ haemolymph	P.d. cells/ haemolymph
<i>Pieris</i> (2)	+42	-23
(3)	+37	-16
	+31	-4
	+47	-12
<i>Tenebrio</i> (1)	+58	-16
(2)	+30	-20
(3)	+54	-25
<i>Dytiscus</i> (1)	+23	-45
	+27	-18
	+17	-18
	+21	-10
(2)	+18	-6
	+28	-7
	+23	-47
	+23	-41

Table 2 shows that in all cases except two, *Locusta* and *Rhodnius*, the interior of the tubule is positive with respect to the haemolymph. There does not appear to be any obvious correlation between the p.d. and the concentrations of sodium and

potassium in haemolymph and urine, and in the case of *Aedes* it is to be noted that the p.d. is not greatly influenced by the medium to which the insect is adapted.

In order to decide whether the differences in concentration can be accounted for by passive diffusion, or whether it is necessary to postulate some process of active transport, it is convenient to express the concentration ratios in terms of p.d., using the relation

$$E = \frac{RT}{nF} \log_e \frac{C_1}{C_2},$$

or, for a monovalent cation,

$$E = -58 \log_{10} \frac{C_1}{C_2},$$

where E is the p.d. in millivolts, C_1 and C_2 are the concentrations of the ion in the urine and in the haemolymph respectively, and the negative sign indicates that the interior of the tubule is negative with respect to the haemolymph.

Table 4

(The figures are millivolts. For explanation see text.)

Insect	p.d. _{meas.}	p.d. _{eq} (Na)	p.d. _{eq} (K)	p.d. _{eq} (Na) - p.d. _{meas.}	p.d. _{eq} (K) - p.d. _{meas.}
<i>Locusta</i>	-16	+5	-46	+21	-30
<i>Dixippus</i>	+21	+23	-55	+2	-76
<i>Pieris</i>	+28	+9	-45	-19	-73
<i>Tenebrio</i>	+45	+36	-47	-9	-92
<i>Dytiscus</i>	+22	+29	-86	+7	-108
Tabanid	—	+45	-87	—	—
<i>Rhodnius</i>	-35	+7	-72	+42	-37
<i>Aedes</i>					
Dist. water adapted	+21	+32	-85	+11	-106
NaCl adapted	+13	+12	-86	-1	-99
KCl adapted	+16	+34	-79	+18	-95

Thus, for example, if C_1 is 100 m. equiv/l. and C_2 is 10 m. equiv/l. and if the system is equilibrium then the p.d. should be -58 mV. If the p.d. is measured and is found to be less than 58 mV. negative, or if it is positive, then the concentration difference $C_1 - C_2$ must be brought about by active transport.

In preparing Table 4 the average values of C_1/C_2 in Table 1 have been used to calculate the p.d. corresponding to the condition of equilibrium (p.d._{eq}). The average value of the p.d. measured (p.d._{meas.}) is obtained from Table 2 and is subtracted algebraically from p.d._{eq}, and if the sign of the difference p.d._{eq} - p.d._{meas.} is negative then active transport is to be assumed.

Taking first the case of potassium, it is obvious that for those insects in which p.d._{meas.} is positive the presentation of figures in Table 4 is superfluous; potassium must be actively transported across the wall of the tubule. In the cases of *Locusta* and *Rhodnius*, although the differences are negative, some assurance is needed that the figures are significant in relation to the scatter of the observations. The 't'

test for significance has been applied, and in both cases the difference is found to be highly significant ($P \ll 0.01$). It is therefore concluded that in all cases in which the necessary measurements have been made there is active transport of potassium; and in the case of the Tabanid active transport is highly probable since $p.d._{eq}$ is -87 mV., the highest value recorded.

In the case of sodium the difference $p.d._{eq} - p.d._{meas.}$ is in general more positive than for potassium. Having regard to the scatter of the observations the only case of a negative difference which merits consideration is that of *Pieris*, -19 mV., and here the concentrations of sodium are so small in relation to the errors of analysis that little significance attaches to this figure. There are insufficient grounds for assuming active transport of sodium; in fact, it is admissible as a working hypothesis that the differences in concentration of sodium are brought about by passive diffusion.

DISCUSSION

The main conclusion which can be drawn from the results just described is that in all the insects studied potassium is actively secreted into the tubule. The question now arises as to how far this conclusion is of general application. Although only eight insects have been investigated: (1) they include representatives of five orders (Orthoptera, Hemiptera, Lepidoptera, Coleoptera, Diptera); (2) they include terrestrial insects (*Locusta*, *Dixippus*, *Pieris*, *Tenebrio*, *Rhodnius*) and aquatic insects (*Dytiscus*, *Aedes*, Tabanid); (3) they include insects having a variety of feeding habits—omnivorous (*Locusta*, *Tenebrio*), carnivorous (*Dytiscus*, Tabanid), herbivorous (*Dixippus*, *Pieris*), detritus feeder (*Aedes*), blood-sucker (*Rhodnius*); (4) they include insects having cryptonephridial systems (*Pieris*, *Tenebrio*).

It therefore seems reasonable to suggest that if future work brings to light other insects which do not actively secrete potassium, these will prove to be the exceptions to a general rule.

In the Introduction the suggestion was made that the active secretion of potassium into the tubule—and its subsequent reabsorption into the haemolymph, which is known to occur in *Rhodnius* and *Aedes*—might be an essential process in the formation of urine. It seems possible that the active secretion of potassium, accompanied by some anion, might produce a high osmotic pressure in the tubule which would cause water to pass inwards through the wall; and that this in its turn would promote a passive diffusion of sodium into the tubule. This hypothesis has the attraction of simplicity, but very little further evidence can be adduced in its support. In the course of this work some measurements were made of the freezing-point depression of haemolymph and urine. It was found that in general the osmotic pressure of the urine did not differ widely from that of the haemolymph, but it could be either greater or less, even in different tubules of the same animal. The possibility of some slight concentration or dilution of the haemolymph during the course of these experiments was not rigorously excluded in all cases, and it was decided not to report these observations in detail in this paper.

It is not to be forgotten that the Malpighian tubules are able to concentrate other substances, e.g. dyes (Lison, 1942 and earlier papers) and that they are able to

eliminate discrete masses of insoluble substances, e.g. biliverdin (Wigglesworth, 1943). When one reflects upon the lack of information concerning the composition not only of the urine of insects, but also of the haemolymph, in which organic substances often predominate, it becomes clear that to put forward any comprehensive theory of urine formation would be premature. What appears to be needed is a more detailed knowledge of the process in at least one insect, and in the immediate future work will be directed towards this end.

SUMMARY

1. The concentrations of sodium and potassium in the haemolymph and in the urine have been measured in eight species of insect.

2. The concentration of potassium in the urine is always greater than in the haemolymph. The concentration of sodium in the urine is generally less than in the haemolymph.

3. In seven of the species the difference of electrical potential across the wall of the tubule has also been measured.

4. In these seven species the results lead to the conclusion that potassium is actively secreted into the tubule. It is very probable that the same is true in the eighth case.

5. It seems likely that the excretion of sodium can be brought about by passive diffusion into the tubule.

I wish to thank Lord Rothschild for advice on the method of measuring p.d. and for the loan of a pH meter; Prof. A. L. Hodgkin for advice and for reading this paper in draft; Miss R. Eccles for practical assistance in the early stages of the p.d. measurements.

APPENDIX I

Locusta migratoria migratorioides R. & L., adult (Orthoptera, Acridiidae). A general account has been given by Schindler (1878). There are about 100 tubules, among which he distinguishes two kinds, yellow and white. I have not observed such differences in this species, all the tubules being brownish red in colour. They are of considerable length and are present as loose coils in this posterior part of the abdomen. The pigment makes it difficult to see the exact position of the point of the pipette after its insertion.

Dixippus morosus (al. *Carausius morosus*), adult (Orthoptera, Phasmidae). The tubules are described by de Sinéty (1901). They are numerous and are of three kinds: (1) 'superior' tubules, opening at the annulus between mid-gut and hind-gut, making a short forward loop and running back to end blindly close to the posterior region of the hind-gut. The 'superior' tubules are of uniform appearance throughout their length and are supplied by small branches of the tracheal system reaching them at various points. They are of relatively large diameter and have transparent walls. (2) 'Inferior' tubules, opening at the annulus in pairs and running directly backwards. Their distal regions are dilated and filled with a milky fluid and they terminate in clumps of cells (cells of Sidorot) embedded in the fat body. Each tube has its own trachea which accompanies it over most of its length. Over their non-dilated regions they present the same appearance as the 'superior' tubules. (3) 'Appendices of the mid-gut.' These appear to be very thin tubules opening separately

into the mid-gut and running back over the hind-gut. The 'superior' and 'inferior' tubules are ideal for operations; no attempt has been made to operate on tubules of the third kind.

Pieris brassicae L., larva (Lepidoptera, Pieridae). The tubules of *Vanessa urticae* are described in detail by Henson (1937), who states that his description also applies to *Pieris brassicae*. There are six tubules arising three on each side from a small pulsatile bladder which is derived from the hind-gut. Thin-walled tubules run forwards over the mid-gut and then turn backwards, their walls becoming thicker, and after some convolutions they pass beneath the muscle layer of the rectum and end in a 'cryptonephridial chamber'. Urine was collected from the thin-walled portions near the bladder. Measurements of p.d. were made on these portions and also on the thick-walled portions. The cryptonephridial region has not been investigated.

Dytiscus marginalis L., adult (Coleoptera, Dytiscidae). Described by Rungius (1911). There are four tubules of considerable length, but their walls are brown and opaque and for this reason it is impossible to collect urine except from a short transparent region near the opening into the gut. Several attempts were made but only one was successful in obtaining sufficient urine for analysis. The p.d. was measured in the transparent region and attempts were also made, 'blind', to insert the pipette electrode into the lumen of the opaque region. There is no cryptonephridism in *Dytiscus*.

Tenebrio molitor L., larva (Coleoptera, Tenebrionidae). A very complete description is given by Poll (1934). There are six tubules. Near their opening into the gut they are transparent, but over most of their course through the body cavity they are brownish and rather opaque. They are gathered together into a common trunk and enter the cryptonephridial region where they spread out over the walls of the rectum. Collections of urine were made from the transparent region and measurements of p.d. were made on the pigmented region.

Tabanid larva (Diptera, Tabanidae). Two of these larvae were brought in by a collector. Preliminary dissection of one of them showed that it was very suitable for operation; the other was used for the collection of urine. No measurement of p.d. was attempted. There are four tubules, briefly described by Stammer (1934), which run freely in the body cavity. Over most of their length their walls contain a brownish pigment which is not so dense as to make operation difficult.

Aedes aegypti L., larva (Diptera, Culicidae). Described by Wigglesworth (1933). There are five short tubules, their blind ends being closely applied to the rectum. They are opaque except just before they open into the gut. As already mentioned, the urine was collected from the intestine. It was not possible to operate on the tubules *in situ*, and p.d. had to be measured on preparations torn open upon a slide under liquid paraffin.

Rhodnius prolixus Stål., adult (Hemiptera, Reduviidae). Very fully described by Wigglesworth (1931). There are four tubules. Each tubule enters the rectum through a specialized region, the ampulla. The proximal portion has transparent walls with a brush border ('bürstensaum') internally and appears to be the site of precipitation of uric acid and reabsorption of water. The distal portion has slightly opaque walls with a honeycomb border ('wabensaum') and it is here that the urine is formed. The tubules are relatively long and convoluted and end blindly near the rectum. Immediately after the animal is fed there is a period of rapid diuresis during which the greater part of the water and salts taken in with the blood-meal is eliminated. In the present experiments urine was collected from the distal portion of the tubule, and the measurements of p.d. were also made on this portion, during the period of diuresis.

APPENDIX II

Briefly, the technical problem of operation is as follows. The wall of the tubule can readily be penetrated by a fine-pointed pipette (5μ diameter) but the finer the orifice the more likely it is to become blocked. If a wide-mouthed (30μ diameter) pipette is used the force required to penetrate is greater; the tubule is stretched and is usually dragged into an unfavourable position. To penetrate with a wide-mouthed pipette it is necessary to hold the tubule firmly and to apply the pipette as close as possible to the place at which the tubule is held. This calls for the use of forceps with very fine points. For this purpose ordinary steel forceps are unsuitable because the points become exceedingly delicate when ground sufficiently thin. This difficulty is overcome by using forceps tipped with tungsten.

The blades of the forceps (Fig. 1*a*) are made from strips of steel about $2.5 \times 0.2 \times 0.02$ in. (old blades of the miniature 6 in. hacksaw are used). They can be spot-welded together at the base, but it is more convenient to bolt them to a small block of brass since this makes possible a final adjustment to bring the points together. A piece of tungsten wire 0.5 in. long and 0.02 in. in diameter is brazed to the tip of each blade (Fig. 1*b*). The tungsten

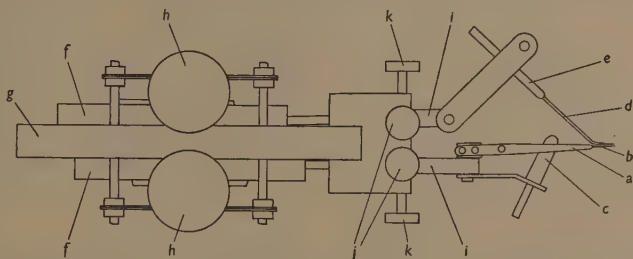


Fig. 1. Micromanipulator assembly carrying pipette and forceps. *a*, blades of forceps; *b*, tungsten tips of forceps; *c*, rubber bulb between blades of forceps; *d*, silica pipette; *e*, wider tube into which pipette is sealed; *f*, bar taking fore and aft movement; *g*, bed upon which bar slides; *h*, adjustment controlling fore and aft movement; *i*, square-section bar spring-mounted on bar *f*; *j*, adjustment controlling lateral movement; *k*, adjustment controlling vertical movement.

wires are then dipped into molten sodium nitrite and allowed to dissolve slowly until they taper to moderately fine points. The points are then ground by hand upon an Arkansas stone, under a medium power binocular. The 'flats' where the points meet are made by grinding on a thin sheet of bakelite covered with the finest carborundum, the sheet being slipped between the points. An adjusting screw is provided which brings the points together in the 'at rest' position. For separating the points a small rubber bulb (fountain pen reservoir) is mounted between the blades and connected to a rubber tube which is held in the operator's mouth (Fig. 1*c*).

The pipette (Fig. 1*d*) is drawn from thin walled silica tubing of about 1 mm. diameter down to an orifice of about 2μ . A jagged end makes penetration easier and can be produced by crushing the point with fine forceps under the binocular. The final orifice may be as much as 30μ in diameter. The terminal portion is then bent over to an angle of about 30° . Prior to use the pipette is sealed into a wider tube (Fig. 1*e*) and the terminal portion is filled with liquid paraffin stained with Sudan Blue.

It is of course necessary to mount both the forceps and the pipette on micromanipulators, but these do not require to have the high precision of instruments used for operations upon cells. The fore and aft movement is of the rack and pinion style, but operates by friction; a steel bar (Fig. 1*f*) of circular cross-section is made to slide back-

wards and forwards along a brass bed (Fig. 1*g*) by rotation of a handwheel (Fig. 1*h*). The steel bar carries a short length of spring steel wire which joins it to a piece of square-section brass bar (Fig. 1*i*). The lateral and vertical movements are brought about by screws (Fig. 1*j*, *k*) which displace the brass bar against the reaction of the spring. It is convenient to build the two manipulators upon the same framework and the combined instruments are carried on a Palmer adjustable stand.

Nearly all the operations described in this paper were carried out on tubules *in situ*. One of the troubles met with is that light from an external source can be reflected from the numerous curved surfaces of partly exposed organs and the glare from these makes it difficult to see below the surface of the haemolymph. This is overcome by illuminating through a glass rod, about 1 mm. in diameter at the end, which dips into the haemolymph and is equivalent to a submerged source of light.

It is also necessary to protect the preparation from desiccation. This might be arranged by working in a moist chamber, but such a chamber would have to be inconveniently large to accommodate the various pieces of apparatus which are used. In any case, a completely saturated atmosphere is difficult to achieve in a chamber which has to admit the operator's hands and allow them sufficient freedom of movement. A neater method is to generate a stream of saturated air and direct it over the preparation. Compressed air is humidified by bubbling through hot water and is then allowed to pass through a length (about 15 ft.) of tubing so that it can take up the temperature of the room. The air stream leaving the tubing is saturated with water vapour and is if anything slightly above room temperature. When tested with a mirror it shows a slight tendency to produce fogging. This, however, is advantageous rather than otherwise since the heat produced by the animal and by the illuminator will tend to keep the preparation at a slightly higher temperature than the air. Experience has in fact shown that on the whole there is a slight increase in the osmotic pressure of the haemolymph in experiments which last about 1 hr.

The insect is pinned out on a small operating table and the Malpighian tubules are exposed. The illuminator is placed in position. A suitable length of tubule is selected and the manipulator is lowered so that the forceps, with their points held apart, descend upon the tubule and are allowed to grasp it. The point of the pipette is laid upon the tubule just beyond the forceps, is lowered so as to indent the tubule and is then thrust forwards. If it is seen to penetrate air pressure is applied to drive out a droplet of liquid paraffin. The pipette is thrust forwards a second time so that its point lies just beyond the droplet which then serves as a seal to prevent the entry of haemolymph through the wound. It is often necessary to apply slight suction to start the flow of urine into the pipette, but once this has begun capillary forces usually become effective and suction is no longer needed. As the urine rises into the pipette the walls of the tubule can be seen to collapse slightly. Much of the urine momentarily present in the tubule is thus collected at once. If the tubule is secreting actively the urine continues to rise in the pipette and as much as 0.1 cu.mm. may be collected in 15 min. under favourable conditions. If secretion is slow there is very little movement after the initial rise and one has to be content with 0.01 cu.mm. or less. At the end of the period of collection the pipette and forceps are raised together (by the adjustment on the Palmer stand) so as to clear the haemolymph. The vertical adjustment of the pipette mounting is moved so as to raise the pipette slightly, but this merely bends it since the point is still inside the tubule. The pipette is drawn quickly backwards and as it emerges from the tubule it flicks clear of the forceps and there is no time for adherent haemolymph to be drawn into it. The pipette is then removed from the manipulator with all possible speed and the sample is blown out into a watch-glass filled with liquid paraffin. If the watch-glass has previously been varnished with bakelite there is no tendency for the droplet to spread over the surface of the glass.

For the measurement of p.d. a silver wire, coated with silver chloride, is inserted into a pipette which is filled with a saline approximating in composition to the urine, and the pipette is thrust into the tubule in the ordinary way. If necessary a droplet of liquid paraffin can be drawn up into the tip of the pipette and later expelled inside the tubule to form a seal to the wound. The other electrode is a glass tube of wider aperture which dips into the haemolymph; this tube contains a saline of approximately the same composition as the haemolymph, set in agar, and contact with it is made through a chloride-coated silver wire as before. The asymmetry potential is first measured with both electrodes dipping into the haemolymph and is compared with the potential measured when the pipette is inserted into the tubule. The potentials are measured with the valve voltmeter which forms part of the Cambridge Instrument Co. pH meter. Since this instrument will only measure potentials negative with respect to earth a backing-off potential of -500 mV. is applied from a potentiometer in series with the lead to the pipette.

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NAVIGATION IN THE MANX SHEARWATER

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INTRODUCTION

Many species of birds have been shown to home from areas of which they could have had no previous experience, but the results were of a nature that did not afford unequivocal evidence of a true navigational faculty. Wilkinson (1952) has demonstrated that the extensive experiments of Ruppell (1935, 1936, 1937) and of Griffin (1940, 1943) produced results that could be explained simply on a basis of random search for known landmarks. Matthews (1951*b*) was able to demonstrate true, bico-ordinate navigation in homing pigeons. These fulfilled the two essential criteria by giving initial orientation in the home direction and swift returns. Kramer & St Paul (1952) and Kramer (1953) have substantiated these results. With wild birds the only critical evidence of navigation was that obtained by Matthews (1952*a*) with Lesser Black Back Gulls, and that only in respect of the initial orientation. Further experiments were therefore needed to settle the question, and to examine the basis of such navigation.

MATERIAL

The Manx Shearwater (*Puffinus p. puffinus*) breeding on Skokholm Island, Pembrokeshire was the species used. Some account of its breeding biology and migrations has been given by Lockley (1929, 1930, 1942). It has many advantages for this type of work. About 10,000 pairs nest on this small island, in burrows from which they are quite easily extracted. Incubation is shared, each bird taking a spell of several days without feeding, thus allowing transport or experimentation of several days. Being about the size of a pigeon it is easily handled and not fragile. It is very loathe to desert, and will continue sitting for many days if the mate is late in homing. The egg is highly resistant to chilling. The incubation period, and hence the time available for homing experiments, is prolonged, about 7 weeks. Although the species is normally diurnal, the birds only come in to their burrows after dark and watch for returns can thus be limited to 2 or 3 hr. a day.

The recoveries of banded birds from Skokholm are discussed in detail in Appendix A. Any release point well inland will be unknown to them, and only the coasts south of Skokholm down West France to North Spain will be well known. There is some evidence that this shearwater, like many of its relatives, migrates into the opposite (southern) hemisphere in winter, and even when breeding the birds may forage up to 300 miles south. Certainly the birds are capable of long, sustained flights, and would be expected to have any possible navigation mechanism developed to a high degree. It flies either in low, careening glides or with beating

wings, but does not normally soar and hence is suitable for orientation observations. Moreover, when released inland it will have no incentive to linger, being such an exclusively pelagic bird.

The main disadvantages lie in the nocturnal return to the burrow. This means that birds will normally only be recovered at the burrow, but frequent checks during the dark period enabled up to 100% recoveries to be made. The birds may well home to the neighbourhood of the Island several hours before they are able to land, and the apparent homing time would be lengthened accordingly. However, allowance can be made for this or the release time adjusted as described later. During the full-moon period the birds show less inclination to come to land if there is little cloud cover, so such periods have to be avoided in planning releases. A further complicating factor is that birds may be at different stages of their 'shift' when taken for homing, and it is not possible to dig sufficient nests and make sufficient inspections to obviate this. But the very fact that large numbers are being used will help to cancel out any effects, and there is no question of the bird's reserves being used up by the end of the usual shift.

The Skokholm shearwaters had been tested in homing experiments previously, by Lack & Lockley (1938) and Lockley (1942). Forty-one birds were released giving seventeen returns at speeds between 8 and 74 miles per day, with the exceptions of one covering 125 miles in 10 hr. and two (released together) 200 miles in 24 hr. As regards initial orientation '...some birds flew off in the direction of Skokholm, others did not, and the former do not appear to form a significant proportion of the whole'. While these results indicated that homing ability existed in this species they did not provide any more conclusive evidence of navigational faculties than other work, despite the much quoted return from Venice (930 miles in 14 days). They did indicate that further critical experiments might be more successful.

METHODS

Only reasonably short burrows, up to about 6 ft., in firm soil and of a simple nature, were used. Plenty of these were to be found within half a mile of the Bird Observatory buildings. The presence of a shearwater is confirmed by the characteristic smell, and the finer points of the burrow's topography determined by a long, flexible probe. An inspection shaft, rarely more than 18 in. deep, is sunk over or slightly to one side of the nesting chamber. The bird is banded and a stone or slate cover, surmounted by the original sod, is placed over the hole to keep it light- and weather-proof yet easily inspected. Secondary shafts are sometimes necessary along the burrow. The nest's position is marked by a white-topped stake and a numbered peg. 441 occupied nests were so prepared in 1951 and 1952, but seventy-seven of these were held by birds that did not lay an egg (or had lost it prior to excavation). These latter were not used for experiments, but only the actively breeding birds. The single egg would appear to be laid during the first 2 weeks of May, laying is certainly virtually complete by the third week. First hatchings occur in the third week of June and are over by the second week in July. A few days after hatching the

parents leave the young and only visit it briefly during the night. It is then much more difficult to check their return. The great majority of homing birds were taken from an egg, all those up to the end of June. Some fifty-two out of eighty-three further releases up to 11 July had perforce to be done with birds taken from young. For transport, fibreboard boxes, $16 \times 10 \times 10$ in. with a diagonal partition, were used, one bird being placed on either side, bedded on dry bracken as in the nest. Ventilation holes, $\frac{1}{4}$ in. diameter, were punched round the sides near the top. It was generally convenient to collect up twenty birds for a consignment, one per burrow. They were taken by boat 7 miles to Dale Fort, then by van to the railway at Haverfordwest. In most cases it was possible to release the birds within 30 hr. of being placed in the boxes. 338 birds were treated in this way (including sixteen local releases), and the great majority travelled very well and flew strongly on release. Five died in the boxes, but these casualties were sporadic and not proportional to the length of incarceration, one death occurring after only 14 hr.

Release points were chosen in wide open spaces, the birds at Cambridge being released from the University Library tower. The liberators, on whose data the valuable orientation information is based, released the birds one at a time, tossing each one up into the air and following it with binoculars until out of sight before releasing the next in a different direction. The nearest compass point to the bird's position when lost to sight was noted, also the time taken to reach vanishing point. The approximation to compass points (W., W.S.W., S.W., etc.) is considered to be sufficiently accurate and, moreover, was made necessary by the unreliability of compasses on top of a steel frame building. All releases except that at Llandoverly (p. 382) were in full daylight, between 07.00 and 19.00 hr. (all times B.S.T.). The positions of the release points, and the numbers of birds released there, are shown in Fig. 1. They are all in areas that can be considered to be unknown to the birds from Skokholm. Inland releases in England, with two long north/south coast lines, form especially stringent tests of navigation in a normally pelagic bird. If only random wandering was the basis of homing the chances of reaching the wrong coast and then searching along it are equal at Haydon and weighted against the birds at Cambridge. The latter is clearly one of the best possible release points, quite apart from convenience of laboratory accommodation, etc. The other points were necessary to check that orientations were not due to some local peculiarity of topography, or, say, a simple tendency to fly west when released inland. Also when it was possible to use the same birds a second time another release point in a different direction was needed. That planned in Eire could not be brought into operation because of foot-and-mouth restrictions. The release in the open Atlantic was from the Ocean Weather Ship, *Weather Recorder*.

Burrows at which homers were due were visited and inspected several times in rotation from midnight until 2 or 3 o'clock in the morning, while there was yet any traffic among the shearwaters. Homers were marked with a spot of light paint on top of the head, making it easy to distinguish them from their mates, though band numbers were of course checked as well. These regular checks were maintained up to the tenth night from release, or longer if the mate was still sitting. Stragglers

coming in after this period have little interest in a study of navigation, but irregular checks were maintained for as long as possible. The number of nests to be visited per night had to be limited in this way to about forty, otherwise the necessary frequent visits could not be made.

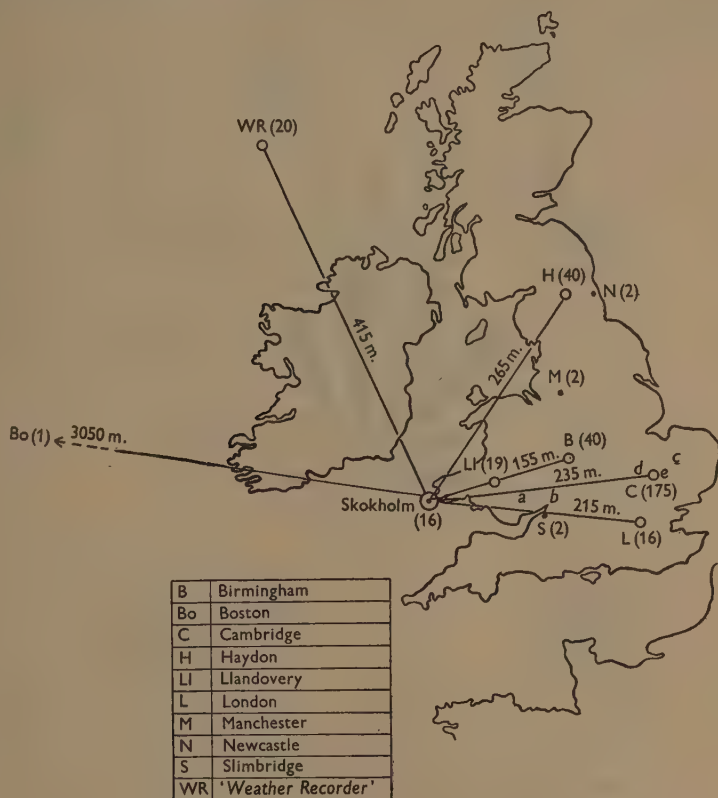


Fig. 1. Location of release points. *Note.* Number of birds released in parenthesis.
a-e recoveries en route.

EVIDENCE OF TRUE, BICO-ORDINATE NAVIGATION

From the data provided by the liberators, radial histograms can be constructed as in Figs. 2 and 3, the length and breadth of the various rays being proportional to the number of birds lost from sight in those directions. In Fig. 2 are collected the releases made under cloudless skies. It will be seen that there is a marked home-ward tendency which becomes very striking when the results are summed in the central figure. The chances of such an orientated distribution being derived from

a chance scatter are quite negligible ($P < 0.001$, as determined by a t test regardless of sign, the method described in Matthews, 1953*a*). Fig. 3 shows the orientations obtained from releases under variable, high thin cloud, with the sun visible throughout. The homeward orientation is only slightly (not significantly) less marked than in Fig. 2, and again cannot be attributed to chance ($P = 0.002$). The time for which the birds were in sight, averaging 3.0 and 3.1 min. respectively, also showed no difference between the two categories.

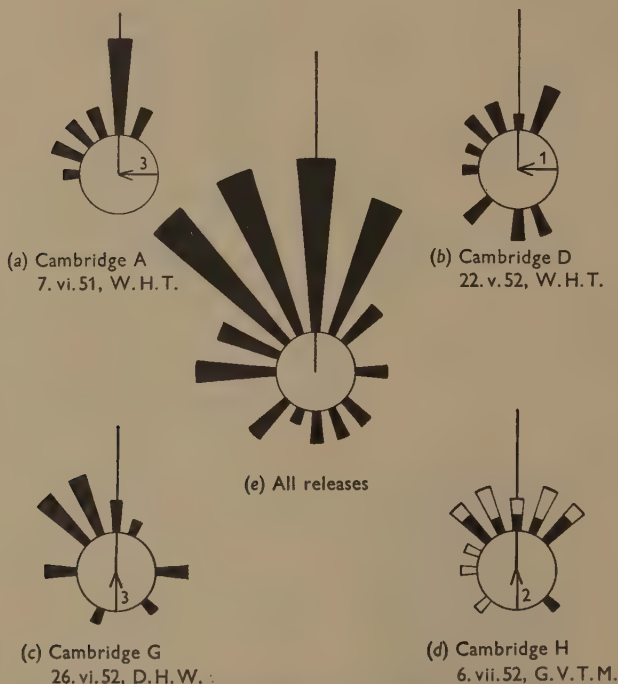


Fig. 2. Initial orientations under cloudless skies. *Note.* Length and breadth of rays proportional to number of birds in that direction. Basic unit—one bird. Home line vertically upwards. Wind direction and Beaufort force shown by arrow. Initials of liberator given—see Acknowledgements.

We have here, then, extremely strong, concrete evidence that the shearwaters were at least roughly orientated towards home within about 3 min. of release, arguing that they must possess some form of navigational mechanism. It is particularly important to note that the results are *repeatable* and are not dependent on any one observer, release point, home direction or wind direction and the orientation is shown both on land and at sea. The results are all the more remarkable when it is remembered that the whole experience of transport and release is

novel and presumably somewhat disturbing to the birds, particularly those finding themselves for the first time out of sight of the sea.

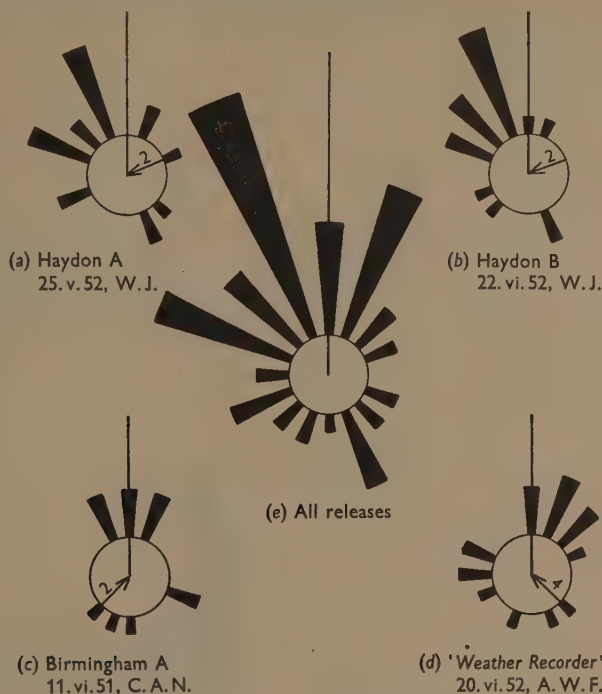


Fig. 3. Initial orientations under lightly clouded skies, sun visible throughout.

The returns achieved from releases under cloudless or lightly clouded skies (including those for which sufficient orientation data are not available, such as birds flying very low and lost in less than a minute behind trees, etc.) are listed in Table 1.

Table 1. Returns from releases under cloudless or lightly clouded skies

Released	Date	No.	Returned on nights										Returned later	Missing
			1	2	3	4	5	6	7	8	9	10		
Cambridge D	22. v. 52	20	2	7	1	.	.	1	.	2	.	.	2	5
Haydon A	25. v. 52	20	6	2	2	2	.	2	.	1	1	1	1	2
Slimbridge	2. vi. 51	2	.	1	1
London A	3. vi. 51	10	2	1	1	1	.	1	.	1	.	.	1	2
Cambridge A	7. vi. 51	15	4	5	2	2	1	1	.
Birmingham A	11. vi. 51	20	2	6	4	1	.	×	×	×	×	×	2	5
W. Recorder	20. vi. 52	20	.	1	5	2	1	2	4	1	.	1	1	2
Haydon B	22. vi. 52	20	.	1	3	1	1	1	.	3	1	.	1	8
Cambridge G	26. vi. 52	19	.	3	.	1	.	1	2	1	×	×	×	11
Totals		146	16	27	19	10	3	8	6	9	2	2	9	35

Note. No watch kept for returns from Cambridge H, or on nights marked ×.

Attention may first be called to the fact that sixteen of these birds released in unknown areas were back in their burrows on Skokholm the *same night*. Dividing the straight-line distance by the time between release and recapture gives them minimal homing speeds:

Released between	At	Miles per hour
07.05 and 07.30 hr.	London A	12.2, 12.2
07.31 and 08.33 hr.	Birmingham A	8.7, 8.8
10.30 and 12.14 hr.	Haydon A	17.4, 17.9, 18.2, 18.5, 19.3, 20.0
15.16 and 15.34 hr.	Cambridge D	21.5, 22.6
18.21 and 18.42 hr.	Cambridge A	28.0, 29.4, 29.4, 35.2

Thus the releases earlier in the day gave lower minimal speeds, from which we may infer that such birds had completed their journey back to Skokholm in daylight and had to wait some hours before they came in to land. Since the flight speed of the shearwater can be taken as around 30 m.p.h. (Lockley, 1942) we have evidence, incontrovertible in some cases and strongly inferential in others, that these birds flew almost directly back to Skokholm. Since they formed a substantial proportion (18%) of their releases, we are clearly not dealing with purely chance happenings. The importance of this evidence, in conjunction with the orientation behaviour, will be clear. For the first time, it has been demonstrated that a species of wild bird *can*, given the correct conditions, fly straight home across a wide stretch of unknown country. Still considering these five releases it will be seen that the proportion of first and second night returns are interrelated, 6 and 2 for Haydon A but 2 and 7 for Cambridge D and so on. By reason of the late starts from Cambridge, instead of the birds having to wait about before landing, only the faster returns will be able to reach Skokholm in time to land, say by 02.30 the next morning. In fact, it seems that only those which reach a well-known strip of coast by dusk would be able to *pilot* their way through the darkness (p. 382). It seems very probable, therefore, that returns on the second night had homed very much faster than the extra 24 hr. elapsed would suggest. The distribution of returns shown in Fig. 4, with 53% back by the second night and 75% by the fourth is clearly very different from that which would be expected on the basis of random search.

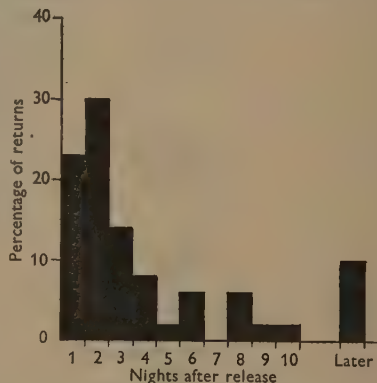


Fig. 4. Distribution of returns from sunny, early releases.

The remaining three releases in Table 1 gave poorer returns, despite the fact that the birds were well orientated on release. That from the *Weather Recorder* may be considered separately since the distance was nearly twice as great, the birds had had 4 nights in the boxes instead of one, and being at sea there would both be less compulsion to keep flying and more temptation to rest and feed. The releases at

Haydon and Cambridge are, however, directly comparable to the earlier ones as in Table 2. The significant differences lie in swift returns and in the missing birds

Table 2. *Returns from sunny releases at 155-265 miles*

Dates of releases	No.	% returning on nights		% returning later	% missing
		1 and 2	3 and 4		
22. v.-11. vi.	85	44	19	21	16
22. vi.-26. vi.	39	10	13	28	49

($P < 0.001$ in both cases), and some seasonal factor must be concerned. All these birds were taken from eggs so there would be no less likelihood of their being checked at the burrow. The embryo is perhaps less resistant to chilling at this late stage, but many of the missing birds' eggs were being covered by the mate, and the fact that an egg is dead or addled does not seem to prevent the birds brooding for at least the normal spell. It would seem unlikely that the birds would desert more readily late in incubation or lose the urge to home. Of nineteen birds released 65 miles away in special circumstances (see p. 382) on 28 June 1952, eleven were back by the second night. Again, six local releases on Skokholm 9 July 1951 in the late afternoon (having been held for 39 hr. in boxes) gave five returns that night, with the last in on the second night. The urge to home would thus appear to be present at this late stage, and the most plausible explanation of the falling off in returns would attribute it to a decline in general stamina.

It is clear, then, that different factors are concerned with orientation and with the return home in shearwaters, much as they are in pigeons (Matthews, 1953*b*). Nevertheless, there is a general association between a good start and a swift return. Birds starting within two points of the home direction gave 33% (of 72) returns on the first and second nights as against 16% (of 49) of those making starts wider of the mark ($P = 0.04$). This suggests that the initial direction of flight is maintained for some time at least, as also do the few recoveries of birds that met with accidents after release. These were found as shown in Table 3. These recoveries are indicated

Table 3. *Recoveries of shearwaters en route from release points*

Bird	Released	Recovered	Lapse (days)	Distance (miles)	Direction of	
					Recovery point	Initial flight
<i>a</i>	Cambridge	Crickhowell	6	150	± 0	± 0
<i>b</i>	Manchester	Tewkesbury	6	100	-2	-2
<i>c</i>	London	Brandon	2	80	+5	+4
<i>d</i>	Cambridge	Eaton Socon	21	15	± 0	+1
<i>e</i>	Cambridge	Newmarket	2	10	± 8	-6

on Fig. 1. Not only is there a strong association between the initial and final bearing, but these recoveries support the evidence that the birds are seeking home directly and not making for the nearest coast. Bird (*a*) is particularly eloquent in

this respect, and with (b) and (d) recalls the recoveries of three starlings not far from the direct line home by Ruppell (1937). These five birds, with another that came to grief soon after release, are included in the 'missing' totals since there is no evidence to show whether others that were missing had not met a similar fate. A considerable proportion do survive, for 27% of those thirty-three 'missing' from 1951 were found in burrows on Skokholm in 1952, as compared with 52% of the fifty-six known to return in 1951 ($P=0.03$).

In pigeons it has been possible to demonstrate wide variation in orientation and homing ability (Matthews, 1951*b*, 1952*b*, 1953*b*), the birds giving consistently good (or bad) performances for a number of releases from different points. This is in itself confirmation of the existence of a true navigational ability. The shearwater offers unusual opportunities for similar individual testing since it has a strong tendency to return to the same burrow year after year. It was not considered feasible to use the same bird twice in the same season because of the increased risk of desertion. Of eighty-nine used on homing tests in 1951 thirty-eight were recovered on Skokholm in 1952, but only twenty-four were in the right breeding state and available when required for testing by release at a second point in a different direction. Because of variations in release conditions, etc., this is not considered a sufficient sample for detailed analysis. This should be possible after the 1953 season when it is hoped to obtain some sixty additional records. Mention may be made, however, of one bird that returned from Cambridge on the first night in 1951, and from Haydon on the first night in 1952 (8.0 and 14.6 hr.).

The array of evidence from initial orientation and swift returns is far more convincing evidence of navigational ability than any isolated homing feat, however remarkable. Against this background one such case can now be presented and properly appreciated. In 1952 two shearwaters were sent by air to Boston, U.S.A. Although the journey only involved 3 nights in the box one died en route, the other was released in good condition in bright sunshine at 13.15 B.S.T. on 3 June. It flew east (seawards). Watch was commenced at the two burrows on 12 June, and on 16 June at 01.30 AX 6587 was back, replacing the mate that had been there an hour earlier. It is interesting to note that confirmation that this bird alone had been released arrived by boat some 10 hr. later! The shortest distance (great circle) between Boston and Skokholm is 3050 miles. This had been covered in $12\frac{1}{2}$ days, a minimal daily average of 244 miles—the equivalent of a Cambridge-Skokholm flight every day. It is by far and away the longest successful homing flight obtained with any species of bird, previous long distance flights being:

Species	Distance (miles)	Miles per day	Reference
White Stork	1400	117	Wodzicki <i>et al.</i> , 1938
Swallow	1150	164	Ruppell, 1937
Alpine Swift	1000	333	Schifferli, 1942
Manx Shearwater	930	66	Lack & Lockley, 1938

Even so only the last was definitely well outside country it might have known on migration. Homing pigeons have returned over 1000 miles, but only after considerable training over part of the course. The minimal speed of AX 6587 was also in excess of most previous flights, even those over much shorter distances. While there is no direct evidence that the bird maintained an undeviating line for home little room is left for diversions and none for random search.

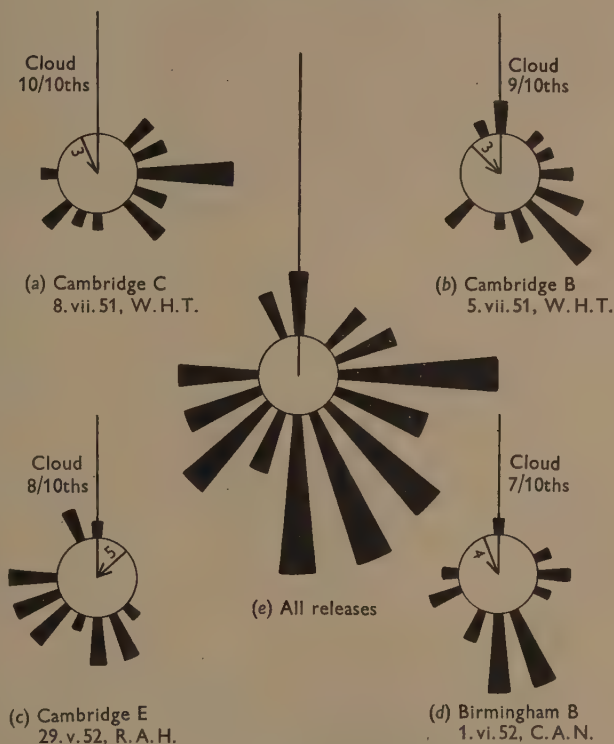


Fig. 5. Initial orientations under heavily clouded skies.

Note. Amounts of cloud are roughly average for period of releases.

THE PHYSICAL BASIS OF THE NAVIGATION

We have seen that shearwaters released in sunny conditions have given very clear evidence of the existence of a navigational ability. Those released under conditions of heavy cloud present a very different picture. Their initial scatters are shown in Fig. 5. Homeward orientation has completely broken down, in fact there is a distinct ($P=0.006$) tendency for the birds to leave in the opposite direction. Examination of the individual release diagrams shows that the wind direction will account for this tendency, i.e. under these conditions the birds are not orientated

and tend to drift down wind. Further evidence of disorientation with heavy cloud is given by the results of an experimental release, Cambridge F (p. 385, Fig. 6). The time the birds were in sight was longer than in the case of sunny releases, the average 4.2 min. being markedly higher ($P < 0.001$) than 3.0 and 3.1 min. for the latter. This can be taken as reflecting a general state of disorientation with the birds hesitating and circling near the release point.

The returns obtained from releases made in cloudy conditions are set out in Table 4. They are seen to be very much poorer than those for sunny conditions (Table 1), but the difference may be exaggerated since clouded releases also show a fall in homing success with the advancing season (Table 5). As with sunny

Table 4. *Returns from releases under heavily clouded skies*

Released	Date	No.	Returned on nights										Returned later	Missing
			1	2	3	4	5	6	7	8	9	10		
Cambridge E	29. v. 52	20	.	3	2	3	.	1	2	.	.	.	2	7
Birmingham B	1. vi. 52	20	.	6	.	2	2	.	×	×	×	×	6	4
Manchester	2. vi. 52	2	.	.	1	1	1
Newcastle	4. vi. 51	2	1	.	.	.
London B	18. vi. 52	6	1	1	.	.	.	3	1
Cambridge B	5. vii. 51	20	2	.	.	2	16
Cambridge C	8. vii. 51	20	.	.	3	1	1	3	.	×	×	×	1	11
Totals		90	.	9	6	6	3	5	3	2	1	.	15	40

Note. No watch kept for returns on nights marked ×.

Table 5. *Returns from clouded releases at 155-265 miles*

Dates of releases	No.	% returning on nights		% returning later	% missing
		1 and 2	3 and 4		
29. v.-18. vi.	50	18	16	40	26
5. vii.-8. vii.	40	0	10	23	67

releases, the later tests gave fewer fast returns and more were missing ($P < 0.001$ in both cases). As the late clouded releases were well after the late sunny ones, and twenty-two of the birds of the former were taken from chicks, with less chance of being checked on return, it is probably best to compare the effect of release conditions only among the earlier ones. Comparing these in Tables 2 and 5 it will be seen that sunny releases gave a higher proportion of quick returns than the clouded releases, the latter showing a higher proportion of late returns (44% *v.* 18%, 21% *v.* 40%, $P = 0.02$ in both cases). The bad starts in clouded conditions had the effect of slowing up returns without significantly lowering the proportion finally reaching home. The effects of a bad start are carried over into homing success despite the possibility of subsequent orientation as and when cloud cover was reduced.

This disorientating effect of heavy cloud is precisely the same as in the case of homing pigeons (Matthews, 1953*a*) and Lesser Black Back Gulls (Matthews,

1952*a*). Now that parallel evidence of this nature has been obtained for three species of very different habits, the suggestion that a form of sun navigation (Matthews, 1951*a, b*) is concerned would seem to be very plausible. The most likely form that such navigation would take has been discussed in detail by Matthews (1953*a*), to which reference should be made. Stated very briefly, the bird is required to estimate the sun's arc by observation of its movement over a small part of that arc. Measurement of the highest point gives local altitude and noon. Comparison with home altitude and noon gives the difference in latitude and longitude and hence the direction of home. The experimental investigation of this hypothesis has proceeded fruitfully with pigeons, and parallel attempts with shearwaters will be described. It may be noted that complete overcast is apparently not essential for disorientation. This can be incorporated in the hypothesis since very brief glimpses of the sun would not permit the appreciation of its movement. Even with more prolonged appearances, detection of its true movement might be confused by massive clouds moving in close proximity.

EFFECT OF THE INCUBATION CYCLE ON HOMING

Before passing to the experimental investigation of the sun navigation hypothesis, a further factor which can affect homing may be considered. As explained earlier, it was not possible, in view of the large numbers of birds being used, to dig a large surplus of burrows and check each daily to ensure that all birds were sent off when they had had the same amount of time on the egg. This would in any case run counter to the policy of disturbing the birds as little as possible before and after the homing test, and would increase the risk of desertion before the birds could be used. In any case, any differences in performance resulting from difference in incubation cycles should cancel out if large numbers are used. A check of this assumption can be made from the data collected when inspecting the nests for returning homers.

There do not appear to be any marked fluctuations in the nocturnal visitations for the island as a whole (except in connexion with the full-moon period), and we can assume that change-over of the individual pairs is independent of those of their neighbours. On any one night the proportion of change-overs should be constant and depend on the average length of the incubation shift. The arrival of the relieving bird is a good indication of the end of the sitting bird's shift, though individuals may be overkeen to brood and arrive early, and others may arrive late. The burrows are inspected on the first night after release; for those listed in Tables 1 and 4 this is the second night after the removal of the homer, except for the *Weather Recorder* release. In 40% of cases the mate was found to have arrived, corresponding to a 5-night incubation shift. As some mates may have returned on the night following removal of the homer, and not remained in the burrow, the actual change-over proportion may well be rather higher, say 50% for the 2 nights, corresponding to a 4-night shift. In these cases the homer was probably on its last, or penultimate, day of its shift when removed. Table 6 demonstrates that the proportion of such birds was practically the same in the various conditions of release that have been discussed earlier, i.e. any effects were distributed equally

throughout and would not affect the conclusions reached. The table also shows that those birds which were probably at an early stage of their shift gave more swift returns than those due for relief. The difference is only of significance in the case of the early releases in sunny conditions ($P=0.01$) and this indicates that the effect is a subsidiary one which only becomes important when the conditions and period of release already give good returns. As further indication of this, the effect is then seen mainly in the returns on the first 2 nights from release, 56% of those early and 27% of those late in their shifts ($P=0.01$). Birds that are due for relief are thus less likely to hurry back than those in which the urge to incubate is still strong.

Table 6. *Effect of incubation cycle on swiftness of return*

Conditions and period	Total	% reliefs	% returned on nights 1-4	
			Early in shift	Late in shift
Sunny, early	85	39	73	45
Sunny, late	39	44	27	24
Clouded, early	50	44	43	23
Clouded, late	40	35	8	14
All	214	40	45	30

INVESTIGATION OF THE SUN-NAVIGATION HYPOTHESIS

The well-known fact that pigeons will not fly through the night, and very rarely after sunset (Matthews, 1953*a*), cannot be cited as strong evidence for sun navigation since the pigeon is so strictly diurnal. The undoubted existence of nocturnal *pilotage* in shearwaters opens a way to test further the hypothesis that their *navigation* is dependent on the sun. Their night vision may not be very good, they tend to blunder into novel obstacles such as the human observer. But they are able to fly direct to the immediate neighbourhood of their burrow and select the right one from scores of others even if the mate is not present to assist such pin-pointing by calling. Indeed, despite the recognizable individuality of the flight calls, the resultant cacophony may be akin to that produced by traffic in eastern cities, expressive of a desire to avoid collision, of nervous tension and general excitement. The first night returns from Cambridge A (p. 376) must have completed their journey in darkness, but they had of course been orientated during daylight.

As will be seen from Appendix A, an inland release point, even at no great distance, will be unknown to the birds. If released there after dark they should only be able to reach home the same night if they had some navigation device *not* dependent on the sun. The local release described on p. 377 showed that no difficulty would result from an unusually close association of the disturbance of capture and boxing with the release point. The test release was made with nineteen birds from a point in the hills near Llandoverly (Fig. 1). To establish the existence of non-sun navigation, nine of these would have to be back the first night, for one or two might be expected to arrive *if* the birds scattered at random and flew steadily in the initial directions. The birds were released in quick succession from 23.30 on 28 June 1952,

and thus had 3 hr. to cover the 65 miles direct to Skokholm. The sun had set some 2 hr. before, the sky was locally overcast, but quite clear at Skokholm. It was not possible to follow the birds for more than a few seconds in this light. Watch for returns was prolonged till dawn, and a confirmatory check made during the day. Not one of the birds returned on the first night. The following night the unusually high total of eleven returns was found at the first check. Thus the birds had survived the night release and maintained the urge to home, but had been unable to complete the short journey in the hours of darkness and to arrive before it was too light for them to land on Skokholm. Two more returns came in up to the sixth night when watch had to be stopped. This experiment has shown that shearwaters are not possessed of any mysterious agency that enables them to *navigate* in the dark, and forms a useful piece of negative evidence for a form of sun-navigation.

Further analysis must of necessity involve the birds being kept away from the sun for a number of days, and the advisability of such incarceration must be considered. The shearwater is one of the few wild birds that could be used because of its habit of spending several days at a time down its dark burrow. The incubation shift varies in length, Lockley (1942) gives 3-5 days as normal extending to 10 days over moonlit periods. During this period the sitting bird does not feed, but subsists on reserves, losing weight in the process. It is improbable that it drinks either, and in view of its pelagic habits it is quite possible that shearwaters, like seals (Irving, Fisher & McIntosh, 1935) obtain all their water requirements from their food. This would be an interesting physiological problem in its own right, as is that of their prolonged fasting. It might be conjectured that a bird taking over incubation just before the full moon period might have laid on extra reserves of fat, but the shift can also be extended without 'warning' during the moonless periods. Thus if we consider those cases in which a homing bird had not returned by the fourth night, and omitting those in which the mate only appeared once or not at all, we find mates sitting continuously for the following number of nights:

Season	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Total
Mid-June	5	8	12	6	8	6	4	3	3	2	2	.	.	1	1	61
Mid-July	9	9	12	7	3	4	4	7	2	.	2	59

i.e. about half the birds would remain sitting voluntarily for more than 5 nights in the absence of their mates, and their tenacity did not alter with the advancing season. These figures are in many cases minimal since they probably included an extra night before the homer was released, when the nest was not inspected, and in a number of cases were terminated by the arrival of the homer or the end of watching. The bird which was still sitting on the sixteenth night was still present on the twenty-second and twenty-third nights, after which it deserted. In view of the above evidence, it would seem reasonable to carry out experiments which involved delaying release until after 4 nights from capture. Two birds were kept in boxes for this period and inspected at intervals. They remained quiescent during the day, at night there would be some scuffling and calling. On release (5 June 1952) they were

still lively and flew well. Further confirmation is afforded by the returns obtained from the *Weather Recorder* (Table 1) after the birds had been confined for the same period. In the experiments described below the birds were taken just before the full-moon period, when they might be expected to be in the best condition. They were sprinkled with water each day and kept in cool, well-ventilated rooms.

It has been demonstrated that pigeons are using the sun's altitude to estimate their north/south displacement (Matthews, 1953*a*). The birds were kept out of sight of sun and sky for 6–9 days in mid-September when the sun's altitude was decreasing rapidly (being near the equinox), and fell in those intervals $2^{\circ} 19'$ and $3^{\circ} 28'$. The pigeons were then released 1° south of the loft and, with few exceptions, were apparently unable to allow for the unobserved decrease in altitude and flew southwards. Control birds, allowed full view of the sun during incarceration, orientated correctly northwards. This method is not easily applicable to shearwaters. First, there is the theoretical objection that, unlike pigeons, periods when they do not see the sun for several days are of frequent occurrence in their lives, and there is much more likelihood of a corrective factor being selected into their navigational mechanism. Secondly, there is the practical difficulty that the period when the birds are available and in good condition for homing work, mid-May to mid-July, is close to the solstice when the daily changes in altitude are minimal. For example, from 2. vii to 6. vii the fall is only $20'$, or the equivalent of 23 miles in latitude. For birds released at Cambridge it would have the effect of depressing the homeward line by less than a third of a compass point, which certainly could not be detected by the present rather crude observational method. Releases to the south would show no effect, and there would be the additional complication that the known area for shearwaters from Skokholm lies in that direction. Use of the birds later in the season would run the risk that a continuation of the decline in stamina would make them much less resistant to prolonged incarceration—they do not remain in the burrow over the day at all and so probably do not lay up food reserves when visiting the chick. Even in mid-August when desertion of the young has begun and ties with the colony loosened, the fall in altitude over 4 days is still only $1^{\circ} 16'$, and any effect would remain very difficult to detect. It seems that with shearwaters this aspect of their navigation can only be investigated by trans-equatorial experiments.

Prolonged incarceration in the homing season will not then produce any observable systematic errors of orientation in latitude. Determination of longitude must essentially be bound up with the estimation of time by some internal physiological 'chronometer'. It was possible that the disturbance of prolonged captivity might upset such a mechanism. Eight birds were therefore kept boxed for 4 days at Cambridge, in a room which the sun did not penetrate directly, with the normal light/dark alternation of day and night. Their orientation on release (Cambridge H, 6 July 1952) was compared with that of ten other birds brought direct from Skokholm and spending only the one night in the boxes. In this and the subsequent experiments the birds were as usual released singly, two controls following two experimentals. The results are shown in Fig. 2 (*d*), the long-duration birds being

shown in black, the short-duration in white. The sky was quite clear at release and an excellent homeward orientation was obtained for both groups with no discernible differences between them. We can therefore conclude that these experimental conditions will have no effect on orientation, either directly by upsetting the navigational mechanism or indirectly by psychological disturbance breaking the urge to home. The good orientation obtained from the *Weather Recorder* is additional evidence for this conclusion (Fig. 3 (d)), the birds there having been in the boxes over 4 nights, though here, of course, there were no controls.

Although a 'chronometer' would presumably have a basis of internal rhythms, it is probable that external 'pace-makers' would play a part in maintaining its synchronization. The experimental alteration of potential 'pace-makers' is therefore a method of attack which can be employed if longitude determination, according to the present hypothesis, is to be disturbed. The most obvious candidate for selection is the daily alternation of light and dark. A 6-day treatment of irregular light/dark periods, accompanied by irregular feeding, produced a random scatter in

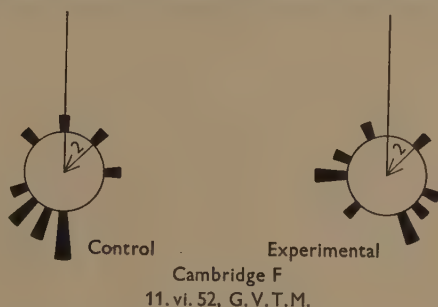


Fig. 6. Initial orientations after passive desynchronization treatment. See text.

pigeons as against orientation by controls (Matthews, 1953*a*). In shearwaters feeding is often very irregular and is unlikely to be a factor in time-keeping, and could not be altered experimentally in any case. The coming of day and of night, however, have a peculiar importance in their lives at the breeding season which should add to their importance as 'pace-makers'.

A first experiment, aimed at producing as little general psychological disturbance as possible, was made by keeping twelve shearwaters boxed for 4 days and nights in a lightproof room which was kept strongly lit continuously throughout the period. For the same time twelve other birds were kept in a separate wing of the laboratory at Cambridge and subject to the normal light/dark alternation, i.e. in the same conditions as the experimental birds in Cambridge H. Unfortunately, weather conditions were not satisfactory for the release on 11 June 1952. The forecast held no promise of improvement, and delay for a further day or two would run the risk of weakening the birds. Accordingly, they had to be released under a heavy cloud cover, 5/10ths increasing rapidly to 9/10ths, as and when the sun appeared in gaps. The results shown in Fig. 6 are quite inconclusive from the present point of view,

although they confirm the disorientating effect of heavy cloud cover even with the sun visible at intervals (p. 381). As we should now expect from a clouded release, returns were slow, by the sixth night only five were back (four were controls). Eventually eight returned from each group (67%). This proportion accords well with that (65%) returning from an earlier clouded release at Cambridge (E. 29 May 1952), and absolves their incarceration from any prolonged ill-effects.

This experiment was a passive attempt to desynchronize the 'chronometers' by the absence of a dark period. The next experiment attempted actively to produce a systematic error by altering the time at which the dark period occurred. While in their burrows, only a dim light will filter to the shearwaters, and the oncoming of night may become apparent less by the change of illumination as by the arrival of the shearwaters from the sea, heralded by their wild clamour. To take this factor into account and complete the illusion that they were still on Skokholm, nine shearwaters were subjected to the following routine during the 4 days and nights that they were in the laboratory at Cambridge. From 20.30 the lights were cut down in the lightproof room until complete darkness was achieved at 21.00.

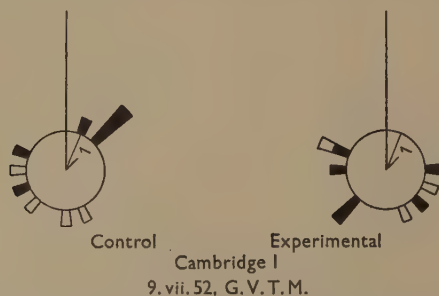


Fig. 7. Initial orientations after active desynchronization treatment. See text.

A record of the nocturnal flight uproar (recorded by Dr Ludwig Koch) was then played until 23.00 when the lights were increased again to full illumination at 23.30. The lights were kept on continuously until 20.30 the next day. 'Night' for these birds thus fell some 3 hr. early, and it was hoped that their 'chronometers' would be similarly put forward. Then on release they would find the sun time well behind their 'chronometer' time, interpret this as being due to displacement *westwards* from Skokholm, and react by orientating to the east. Ten control birds kept for the same period in the separate wing of the laboratory with the normal light/dark sequence should orientate correctly homewards, to the west.

Unfortunately, cloud conditions on the release day, 9 July 1952, were again not satisfactory. At the start there was less than 1/10th cloud and during the following hour nine birds were released. Cloud had increased to 5/10ths by then and was soon about 8/10ths. It showed no signs of clearing so seven birds were released in these conditions when the sun appeared in gaps. The sky then became completely overcast until 3 hr. later when it cleared temporarily to 4/10ths and the last three birds

were released. The results are shown in Fig. 7, the birds released in sunny conditions being shown in black, the others in white. Only the former can give any evidence of the effect of the experimental procedure, and they do show a difference in orientation, in the sense predicted by the hypothesis. But with the small numbers available the difference is not sufficient to be considered significant ($P=0.07$). The experimental birds also took longer to get away, in sunny conditions, than the controls averaging 4.6 min. *v.* 3.4, again this is not significant. Thanks to the vagaries of the English weather this result is inconclusive like the last. But it is certainly more suggestive, and more tests of this nature are required and are planned for the coming season—under blue skies it is hoped.

DISCUSSION OF PREVIOUS WORK

The existence of a true navigational capacity has been demonstrated beyond doubt in the homing pigeon, in the Manx Shearwater and (less certainly) in the Lesser Black Back Gull. How far does the evidence obtained with these three diverse species imply that other birds with homing capacities are likewise equipped? In other words, how far has the lack of any unequivocal evidence of navigational ability been due to inadequate experimentation or the use of incorrect techniques or unsuitable material?

Until recently, far too little attention was paid to the orientation behaviour of homing birds on release. In any case the small numbers used by most workers would preclude any definite decision. In many cases the birds used were unsuitable for such observations, being birds which progress largely by soaring, e.g. Gannets, or small passerines which are likely to perch after release and in any case would be difficult to follow far. Griffin (1943) did analyse the headings of twenty-five Herring Gulls and found no tendency to start in the home direction, but the birds were released in cloudy conditions, and moreover are prone to soar.

The lack of any previous demonstration of straight line homing by a wild bird requires more explanation. The results obtained with the Lesser Black Back Gulls serve as a pointer. It was possible to demonstrate initial orientation, but the return was generally so slow that the second criterion of navigation was not fulfilled. It was suggested that this might be due to the birds adopting their normal, leisurely pace of travel, a conclusion now strengthened by the demonstration that different factors govern orientation and the actual return, in both pigeons and shearwaters. It can therefore be argued that swift returns over considerable distances should only be expected if the species used is one that normally travels fast and far. But only with exactly the right conditions can even the right sort of species be expected to put up the outstanding performance needed. We have seen that with the Manx Shearwater a high proportion of first and second night returns can only be expected when the birds are released: (1) in sunny conditions, (2) relatively early in incubation (before mid-June), (3) near the beginning of an individual incubation shift. The factors are listed in descending order of importance. To them we may add the probable existence of individual variations in orientation and homing powers. Further, the release must not be too distant or too late in the day for the birds to return by the

end of darkness. There are indications that about 300 miles, representing 10 hr. flying, is about the limit for 1 day's flight for shearwaters.

On grounds such as these can we find the explanation for the inability of Lack & Lockley (1938) to demonstrate straight-line homing from unknown country, although using the same species. Of forty-one birds used, nine were taken from empty burrows or simply picked off the ground, and might well have been non-breeders. Then half the experiments were carried out after the third week in June when homing performance would be poorer. Lastly, continued nightly watch at the burrow was not practical for an active farmer. Orientation evidence was lacking because of small numbers and many of the releases took place on the coast whose topography would, at least temporarily, be an over-riding factor. Griffin (1940) failed to obtain evidence of navigation in an allied species, Leach's Petrel. This much smaller petrel, about a tenth the weight of the Manx Shearwater, is much more delicate and easily weakened by transport. Thus after 'nearly 3 days' 50% were in poor condition. Secondly checks for returns were not possible in several cases until the fourth night after liberation, and only late in the season was the procedure of 'inspecting the burrows at regular intervals throughout the nights' adopted. Most of the releases were at sea where it would be difficult to follow so small a bird for long and there were no inland releases.

Previous failures to demonstrate navigation ability in the many species shown to be capable of homing cannot therefore be taken to mean that such ability is lacking. Indeed it would be safer to assume its presence until it is definitely shown to be absent. And since three such very dissimilar birds, pigeon, shearwater and gull, are apparently using the same *type* of navigation, it is presumably of wide-spread occurrence in birds.

APPENDIX A

The range of shearwaters from Skokholm

Thanks to the pioneer work of R. M. Lockley, and years of steady banding by the Skokholm Bird Observatory, some forty thousand of the numbered leg bands issued under the auspices of the British Trust for Ornithology have been put on Skokholm shearwaters. Even so, as would be expected in a bird of pelagic habits, the number of recoveries *away* from the breeding colony have been relatively few, 202 up to June 1952. The great majority of the birds are banded as adults picked up off the ground at night. Large numbers are obtained in this way but the method gives no information as to the individual's age or breeding status. Comparatively small numbers of fledged young are banded, and hence recoveries are mainly of adults, in strong contradistinction to most other species, e.g. gulls (Matthews, 1952a).

The locations of recoveries are indicated in Fig. 8. They emphasize the pelagic nature of the species, there being only nine recoveries inland, of which four are of immature birds. All occurred in September and can be attributed to the birds being blown inland by autumnal gales, as can the inland occurrences of Manx

Shearwaters in general (Kenrick in Lack & Lockley, 1938). It is therefore safe to assume that shearwaters released any distance inland will be in an area unknown to them, and one which they will leave as quickly as possible.

The coastal recoveries are concentrated to the south of Skokholm, south Wales, Devon and Cornwall, west France and north Spain—there being only four isolated recoveries to the north of Skokholm. Only three recoveries came from the English Channel, again immatures in September and probably gale-blown. The east coasts of Britain can thus be classed as unknown to Skokholm shearwaters, and most of the west coasts as probably unknown.

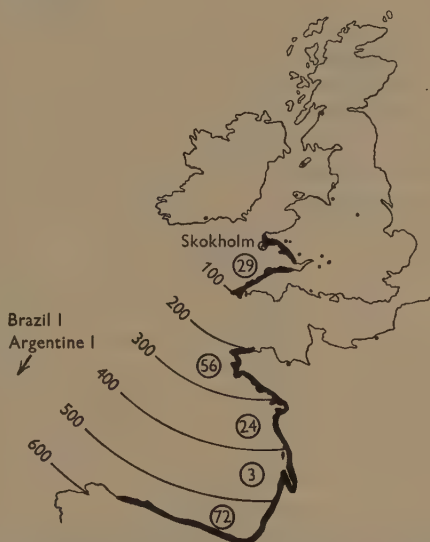


Fig. 8. Distribution of recoveries of banded birds from Skokholm. *Note.* Majority of recoveries along shaded coasts, totals in distance brackets shown in circles. Isolated recoveries shown as dots.

For many years the southwards recoveries terminated abruptly at north Spain, and it seemed that the Manx Shearwater, unlike many of its relatives, had a fairly circumscribed annual range. But there was a complete dearth of recoveries from the beginning of November until February, which suggested that the birds wintered at least well out to sea. Manx Shearwaters had been known to occur regularly off Brazil and Argentina (Murphy, 1936), but as there had been reports of this species breeding in the Bermudas (Reid, 1884; Nichols & Mowbray, 1916; Dwight, 1927) it seemed likely that birds from there or from other undiscovered colonies in, say, the West Indies, would provide the migrants in South American waters. On the eastern side of the Atlantic the species breeds as far south as Maderia and, possibly, the Canaries (Bannerman, 1914), very much closer to South America than is Skokholm. However, in 1951 an immature bird ringed on Skokholm was

recovered only 6 weeks later in October off Rio de Janeiro (Brazil), and since this analysis was made a further report has arrived of an adult ringed in 1947 and recovered 200 miles south of Buenos Aires (Argentina) in October 1952. While further recoveries of this nature must be awaited before we draw definite conclusions it seems probable that the Skokholm population moves much farther south than had previously been suspected. This conclusion is important for the present work in that it places the Manx Shearwater among those trans-oceanic wanderers in which any navigational faculties should be especially highly developed. Occurrences in North America are extremely rare and appear to be only accidental (Dwight, 1923).

Another problem that has a strong bearing on the navigational and flying capacities of the Manx Shearwater is that of its feeding range while incubating. As will be seen from Table 7, there have been a number of recoveries 500-600 miles south of Skokholm during the months May/July when there are eggs or young on the Island. Lockley (1935) reasonably concluded that such recoveries were of late breeders or non-breeders, in view of the indiscriminate banding technique. Later

Table 7. *Distribution of coastal recoveries south of Skokholm*

Distance (miles)	May/July (%)	Aug./Oct. (%)	Nov./Jan. (%)	Feb./Apr. (%)	Total no.
0-100	23	28	.	1	30+1*
(100-200)
200-300	48	33	.	18	58
300-400	4	24	.	10	24
400-500	2	2	.	1	3
500-600	23	13	(100)	70	71+1*
Total no.	52	61	1	72	188

Plus 1, Brazil, 1, Argentina, October.

* No date.

(1942) he abandoned this cautious attitude following the recovery of two birds in this group that had been ringed the same spring in Skokholm. These recoveries, with the others ringed in previous years, were then considered to have 'settled once and for all the truth that our shearwaters are feeding in the farthest corners of the Bay of Biscay at the same time as they have nests and eggs at Skokholm—that they are, in short, flying distances of 600 miles to feed!' But not one of these birds had been banded on a nest with egg or young in that season, or, for that matter, in any other season. A sixth of the occupied burrows prepared for the present study contained birds that visited them irregularly but produced no egg (or had lost it before excavation). An unknown and probably greater number of non-breeders would be unable to obtain a burrow of their own. Lockley himself estimated that 40% of those which produced eggs were unsuccessful in rearing young. The chances of a non-breeding bird being banded by the pick-up technique are thus substantial, and certainly the mere presence of a bird at the colony during the breeding season is no evidence that it is itself actively breeding.

In the course of the present work 724 birds were banded on egg or chick. One was recovered away from Skokholm in May/July. This bird was the mate of a

homer that had failed to return 10 days after release. Desertion of the burrow and chick might well be expected, but cannot be affirmed. Five days after last being checked at the burrow this bird (AX 3263) was recovered at Concarneau, Finistère, on 19 July 1951, 280 miles south. While there is serious doubt as to whether this bird was still persisting in breeding, its recovery is one of a substantial number off Finistère in May/July (Table 7). Again none of the others was definitely breeding, and only two had been ringed the same spring. But it cannot be denied that there is a possibility that active breeders range south across the mouth of the Channel (where we can expect no records) up to 300 miles from home. Again, the comparative importance of recoveries off Finistère may be exaggerated by the shooting by local fishermen, a factor absent in British coastal waters. Table 7 shows a marked hiatus in the May/July recoveries between 300 and 500–600 miles, the group Lockley was primarily concerned to allot to active breeders. Such a distribution suggests that we may be dealing with two classes of birds, e.g. breeders and non-breeders and not with the general spread from the breeding centre.

There have been reports of many thousand Manx Shearwaters passing by day southwards off Cornwall (Wallis, 1924; Thorpe, 1935; Hartley, 1935) and northwards off Ushant, Finistère (Meinertzhagen, 1948) which may be such feeding movements in progress. So far the most spectacular movements have been observed in April, before egg laying has begun and when potential breeders might be ranging farther afield than when actually incubating.

As most birds have been banded and recovered as adults little can be said about the age composition of the population, the age at which the birds first breed and other interesting points. It is hoped that data on adult mortality rates will be extracted from the mass of recoveries that have been made on Skokholm, where in the main colony about 40% of the birds picked up in a night have been banded previously. Some birds used in the homing tests had been banded up to 5 years previously as adults, but the bands generally wear through in this time and banding had come to a stop during the war, so this is by no means the upper age limit.

APPENDIX B

Use of flight-duration recorders

We have seen that well-orientated shearwaters returning on the first or second night will have flown in a more or less direct line from their inland release points to the neighbourhood of Skokholm. We must investigate the performance of such birds as these, and not that of stragglers, if the basis of bird navigation is to be found. It follows that the exact homeward track is now of only minor interest. Wilkinson (1950) had designed a radio-active flight-duration recorder to determine the approximate length of the homeward track, before concrete evidence of true navigation had been obtained. Recorders of this type have been tested extensively on pigeons (Matthews, unpublished) and over a hundred were used on shearwaters in 1951. There was little difficulty in recovering the recorders from returned birds

such as had been met in Lesser Black Back Gulls (Matthews, 1952*a*) and sixty-three recorders were recovered after flights.

Great difficulty was experienced in the matter of waterproofing the recorder, the interior of which must be kept completely dry to prevent the ball-bearing shutter from sticking, or the film emulsion from being damaged. Various waxed and rubberized wrappings were tried out, but only by embedding the recorder wholly in wax within a further capped dural container, liberally sealed with Bostik, could the penetration of water be avoided. This increased the bulk and weight (*c.* 1 g.) of the

Table 8. 'Flight' duration records obtained with Manx Shearwaters

Release point	Time from release to recapture (hr.)	'Flight' time (hr.)
Cambridge	6.7	7.2
Local	7.7	3.5
Local	7.9	4.5
Cambridge	8.0	9.5
Cambridge	8.0	12.0
Cambridge	8.4	11.7
London	17.7	14.8
Birmingham	17.7	22.1
Birmingham	17.8	21.8
Slimbridge	28.5	11.0
Birmingham	30.2	20.4
Cambridge	30.3	15.2
Cambridge	30.3	21.1
Birmingham	30.4	28.6
Cambridge	31.2	25.2
Birmingham	42.3	84.1
Birmingham	42.3	24.5
Slimbridge	52.3	36.1
Cambridge	56.9	36.7
Birmingham	64.6	43.9
Cambridge	78.1	37.4
Cambridge	129.0	166.3

attachment to a point where it could begin to be an encumbrance to a bird of the present size.

The results obtained from the films in successfully waterproofed recorders are shown in Table 8. These are corrected for the theoretical decay of the source strength between the date of flight and that of subsequent calibration for a known time. It will be at once apparent that in a number of cases the 'flight time' exceeded the maximum possible. This was, at least in part, due to false 'flight' recording while the bird was still in the box. This is shown by the readings of recorders removed after the bird had spent a period in the box, before release:

Hours in box	32	30	32	50	30	49	32	32	50	50	50	50
Recorded as 'flight' (%)	2	4	5	8	8	9	10	12	14	21	73	83

The proportion of box time recorded as 'flight' is both substantial and variable, not entirely related to the period of imprisonment, *i.e.* 1 or 2 nights. It is therefore impossible to apply a standard correction. The position of the recorder, stuck with Bostik over the manus on the inside of the wing, cannot be improved upon to give

wider differences in attitude between flight and rest. The 1 mm. steel ball shutter of the original design had already been replaced by a 1.8 mm. lead ball as the heavier shutter had reduced similar false recordings by pigeons in baskets, to some extent. A denser ball might help even more but the diameter cannot be increased further without scaling up the instrument as a whole. In pigeons the problem was avoided by hardening on the wing a permanent bucket into which the recorder could be slipped just before release. But with shearwaters the elaborate waterproofing required that the complete assembly be hardened into place as a unit, and this can only be done when the bird is confined in the box. The only solution would be to attach a second recorder as close as possible to the first for removal before release. This would double the already considerable amount of work involved—the few results given here resulted from the individual counting of 186,000 alpha particle tracks under the microscope.

The false 'flight' record is presumably built up by the birds scuffling about in the box, or resting with one wing up against the side. It is not wholly due to the unnatural circumstances, since birds remaining in the nest also produce 'flight' records:

Hours in burrow Recorded as 'flight' (%)	34 1	63 5	34 12
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This would also have to be allowed for if there was any delay in recapturing the bird on its return. And if so much 'flight' is recorded while the bird sits quietly in its burrow, how much more will be recorded when resting, preening or feeding on the water? Further, the shearwaters that assemble on the sea off Skokholm from 19.00 onwards do not sit quietly and wait for night to fall. They frequently take off and fly for several minutes at a time. The total amount of flying done in this way is very variable, but is estimated to be between 1 and 2 hr.

Lastly, it has now been found that the apparent reduction in source strength is variable and in most cases faster than would be predicted theoretically, i.e. the 'flight' times in Table 8 would be too high in any case. This discrepancy is not due to flight conditions alone as it occurs when the recorders are standing in the laboratory. This is a problem in radio-chemistry, which might be solved by using a source other than polonium and/or improving the present method of electrolytic deposition on silver foil from dilute nitric acid solution.

SUMMARY

1. Homing experiments were carried out with 338 Manx Shearwaters from Skokholm Bird Observatory, Pembrokeshire. The especial advantages of this species for such work are described.

2. With sunny conditions, strong homeward orientation was shown by birds released at points unknown to them. Early in the season these releases gave very fast, complete returns, more than half back on the first 2 nights. Those on the first night were unequivocal evidence of long, direct flights over unknown country.

3. One shearwater homed at least 3050 miles across the Atlantic in 12½ days.

4. There was a decline in the proportion of returns later in the season, probably due to a loss of stamina.
5. With conditions of heavy cloud the homeward orientation broke down and returns were poorer.
6. The stage of the individual incubation shift has some effect on the homing performance.
7. For the first time we have a demonstration of complete evidence for navigational ability in a wild bird. The suggestion of a form of sun navigation is in line with evidence obtained with pigeons and gulls. The failure of workers with other species to produce such evidence is not considered to weigh against the possibility of navigational ability of this kind in all proven homers.
8. The sun-navigation hypothesis is investigated. Although shearwaters fly to the burrows after dark they are unable to navigate after sundown. Determination of the latitude from the sun's altitude can only be tested in this species by trans-equatorial experiments.
9. Determination of longitude by time differences was tested by attempting to desynchronize the necessary internal 'chronometers'. The first attempt was inconclusive owing to cloudy conditions at release. A second attempt was also partially thwarted by the weather, but gave suggestive results favouring the hypothesis. More experiments of this nature are required.
10. An analysis of the range of shearwaters from the colony, based on banding results, is given.
11. The Wilkinson flight-duration recorder was used on this species, but did not give satisfactory results.

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A POLARIZED LIGHT ANALYSIS OF THE HUMAN RED CELL GHOST

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(With Plate 11)

INTRODUCTION

The structure and composition of the mammalian red blood cell ghost has been the subject of many investigations, mainly because the ghost is the only cell membrane which can be isolated in large quantities, and in what is thought to be a more or less intact condition. A summary of some of the earlier results on its thickness and composition will be given later. It suffices for the moment to say that the mammalian ghost is almost entirely composed of lipids and protein, and that there is probably enough lipid to form a continuous layer at the surface about 40 Å. thick (2 molecules), and enough protein to form a layer, when dry, from 50 to 150 Å. thick.

With one exception, however, there had been no measurements of the thickness of the ghost membrane in the normal hydrated state, when this work was started. *A priori*, it seemed quite possible that the membrane, and especially the protein of the membrane, would be considerably hydrated, since most cells contain from 60 to 90% of water. This was confirmed by the measurement mentioned above; the estimate of Seifriz (1927), by direct observation under the microscope, that the thickness of the membrane of amphibian red cells was 6000–8000 Å. Amphibian red cells, however, differ from mammalian red cells in size, shape and the presence of nucleus, so it seemed desirable to try to find whether mammalian ghosts showed a comparable degree of hydration.

A more important reason for trying to measure the thickness of the wet ghost membrane arose out of the work of Schmitt, Bear & Ponder (1936, 1938). They examined rabbit ghosts in polarized light and found that in glycerol the spherical ghosts showed negative tangential birefringence in their membranes.† They believed originally that this birefringence was due to radially oriented lipid molecules, and also that its strength indicated that it must be caused by lipid layers a great deal thicker than two molecules. Subsequently, they changed their views and

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† In a membrane with a radial optical axis, like the ghosts, it is a matter of convention whether the sign of birefringence is referred to the tangent or the radius (positive with respect to the radius = negative with respect to the tangent, and vice versa). The sign is given with respect to the tangent throughout this paper since it stresses the similarity between membranes and fibres or long plates. Extended protein chains would be positive in all these cases.

stated that this strength of birefringence could be caused by a few layers of lipids (footnote at the end of Harvey & Danielli, 1938). This was correct as regards the strength of birefringence of the ghost membrane, but it was still true that the thickness of the birefringent membrane seen down the microscope appeared to be much greater than a few molecular layers. Recent work, however, had established beyond doubt that there was only enough lipid in a ghost to form a continuous layer a few molecules in thickness, so that if this impression of a relatively thick membrane were confirmed, Schmitt *et al's* interpretation could not be correct.

The measurements described in this paper have confirmed that the birefringent ghost membrane in glycerol is relatively thick—about 0.5μ . A substantial portion of the birefringence must therefore be due mainly to protein, and, since the sign of the birefringence is negative, this protein must have a predominant *radial* orientation. This implies a new concept of the protein arrangement in the membrane.

A preliminary account of this work was published some years ago (Mitchison, 1950). This full account, however, has been delayed owing to the difficulty of getting the final computation of the diffraction formulae. In the interval Ponder (1951) has produced convincing evidence by another method that ghosts are thick-walled, or possibly solid, structures. He has shown that ghosts, when heated, break down into fragments whose total volume is approximately the same as that of the intact ghosts.

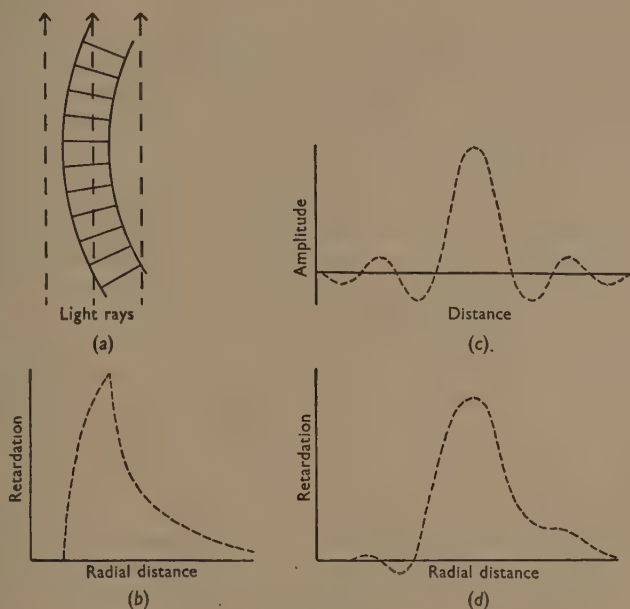
AN OPTICAL METHOD OF MEASURING MEMBRANE THICKNESS

The thickness of a spherical or cylindrical membrane cannot be measured directly under the microscope if this thickness is near or below the limit of resolution. The main reason for this is that diffraction obscures the inner and outer edges of the membrane. Under certain circumstances, however, there is a pattern of light intensity at the edge which can be measured. The thickness of the membrane can then be found by comparing this measured pattern with other patterns calculated from theory for membranes of varying thickness, and seeing which of these gives the best fit.

If a membrane absorbs light (e.g. a dyed membrane) or appears to emit it (e.g. a birefringent membrane), it will have a pattern of light intensity at the edge. It is relatively easy to photograph this pattern, measure the density of the photograph at various points and then express the pattern as a curve of light intensity (or retardation, in a polarizing microscope) against radial distance. The difficulty, however, comes in calculating the appropriate theoretical curves with which the measured curve can be compared. A general description of how this is done is given below, but the detailed formulae can be found in Appendix 1.

The first part of the problem is to derive the 'undiffracted' curve which gives the light intensity or retardation from the object before the pattern has been 'diffracted' by the microscope objective. Let us consider a spherical, weakly birefringent membrane (with a radial optical axis) in a medium of the same

refractive index (to eliminate reflexion and refraction effects) and illuminated by a parallel beam of light in a polarizing microscope (Text-fig. 1*a*). The retardation of a ray which has passed through the membrane will be the product of the coefficient of birefringence and the thickness of membrane traversed by the ray (as in any birefringent object); but there will also be a factor which allows for the varying orientation of the birefringent elements at different points in the membrane. It is convenient to combine this factor with the actual geometrical thickness of membrane traversed, so as to give a term which can be called the 'effective thickness'. The



Text-fig. 1. (a) Spherical birefringent membrane with radial optical axis. (b) An 'undiffracted' retardation curve. (c) A point-diffraction pattern. (d) A 'diffracted' retardation curve.

'effective thickness' multiplied by the coefficient of birefringence gives the retardation. For a membrane of given radius and thickness, the retardation for any ray can be calculated from equation (1) in Appendix 1 (a) and an 'undiffracted' retardation curve can then be drawn (Text-fig. 1*b*). It should be noted that since the coefficient of birefringence is a simple multiplying term it will only affect the height of the final retardation curve, and not its shape.

The second part of the problem is to take diffraction into account, and so to derive a 'diffracted' curve from the 'undiffracted' one. A point object in focus under a microscope gives a diffraction pattern in the image which is a function of

the objective aperture and the wavelength of the light (Text-fig. 1 c). If the point is out of focus, the diffraction pattern depends not only on the objective aperture and the wave-length but also on the distance out of focus. The whole object can be regarded as a large number of points, and therefore the whole image can be constructed by summing a large number of point-diffraction patterns. Thus the diffracted retardation curve can be constructed by substituting a diffraction pattern for every point on the undiffracted retardation curve, and then summing all the patterns. The resulting diffracted curve (Text-fig. 1 d) is broader and flatter than the undiffracted curve, though the areas underneath them are the same. A diffracted retardation curve, derived in this way, is the appropriate theoretical curve for comparing with the measured curve of a birefringent membrane.*

This method involves a number of optical assumptions which are considered in Appendix 2. There are, however, certain general points which are worth mentioning here. The advantage of the method is that it can be used to measure the thickness of a membrane which is near the limit of resolution. If the membrane is thick the method, though applicable, is usually unnecessary. It has, however, a number of limitations. First, the membrane must be spherical or cylindrical. Secondly, the equations in Appendix 1 will not be correct if the medium on either side of the membrane affects the light intensity, though it might be possible to allow for this. Thirdly, the equations only apply to a membrane of uniform structure throughout its thickness. It is possible to modify the equations to allow for a membrane of varying structure, but only when this variation is known and can be expressed quantitatively (e.g. an orientation falling off as the square of the distance from the edge). Fourthly, the surrounding medium must have nearly the same refractive index as the membrane, since otherwise difficulties with reflexion and refraction will arise.

The equations only apply to birefringent membranes, but it is quite easy to write down similar ones which apply to light-absorbing membranes under an ordinary microscope. The practical difficulty of using them to measure thickness is that the contrast from absorption is usually too low to measure accurately, owing to the bright background. With a polarizing microscope, on the other hand, the background brightness is so low that a birefringent object can have a very high contrast.

MEASUREMENTS ON RED CELL GHOSTS IN GLYCEROL

One volume of human blood was mixed with about a hundred volumes of glycerol. The glycerol haemolysed the red cells and also acted as an immersion liquid of high refractive index. The resulting ghosts were smooth spheres with an external diameter of 6.0μ , and they showed a negative birefringence at the edge (Pl. 1 a). A drop of the suspension of ghosts in glycerol was then mounted in a small cell made by cutting a 1 mm. hole in a no. 0 cover-slip with a diamond, waxing the

* In the calculation in this paper, line diffraction patterns were used rather than point patterns, since this simplifies the computation. This is discussed in Appendix 1.

cover-slip to a slide, and then covering with another no. 0 cover-slip. It was important to ensure that there was no movement while the ghost was being photographed. If the ghost drifted this showed up in a photograph as an obvious broadening and blurring of the edge in the direction of movement. There was no sign, however, of this after the ghosts had been in the cell for half an hour. This was confirmed by finding no drift of the ghosts over a period of minutes under a micrometer eyepiece which would have shown movements down to about 0.1μ . There was also no detectable brownian movement. A number of the ghosts were then photographed under a sensitive polarizing microscope (Swann & Mitchison, 1950). Careful focusing was necessary, but this was not difficult since the pattern at the edge of the ghost broadens quickly on either side of the focus, and it was relatively easy to focus the narrowest and sharpest pattern. The details of the optical system were as follows: Cooke, Troughton and Simms polarizing microscope with double thickness polaroids; $\times 95$ 'bloomed' achromatic O.I. objective N.A. 1.30, with an internal stop reducing the N.A. to 1.0 (measured with an apertometer); $\times 10$ compensating eyepiece; two lens 'bloomed' Abbe condenser stopped down to N.A. 0.25, and oiled to the slide; $\lambda/117$ mica plate compensator used 5° from its zero position, giving a background retardation of $\lambda/672$;* 5 amp. carbon arc with Kohler illumination and a narrow iris; exposures of 1 min. on Kodak Plus-X 35 mm. film; magnification of $\times 180$ on the film. Each ghost was positioned in the centre of the field before being photographed. A series of calibration photographs were also taken on the same length of film. These were taken with no ghosts in the field and with varying amounts of compensation, so as to give the film density for known retardations.

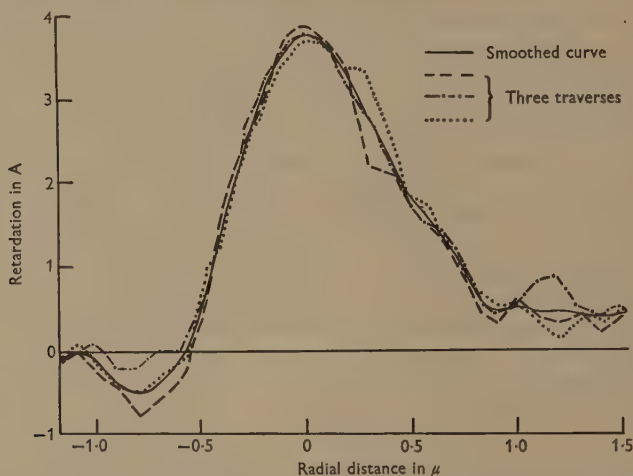
The size of the ghosts on the negatives was too small for measurement, so the negatives were enlarged upon lantern plates to give a total magnification of $\times 1000$ (i.e. the diameter of each ghost was 6 mm.). The calibration negatives were also enlarged, and all the plates were processed together.

The plates were measured with a densitometer designed to work with small areas of film (Swann & Mitchison, 1950). Readings were taken along a radial line traversing the membrane edge. The lightest part of one of the light quadrants of the membrane was chosen, where the tangent to the membrane was at 45° to the polarizer, and where the retardation of the compensator added to that of the membrane. Readings were taken on 100μ square areas of plate every 100μ along the traverse ($=0.1\mu$ on the ghost). Three traverses were taken in each of the two light quadrants of eight ghosts, each of the three traverses being separated by a tangential distance of 200μ on the plate.

Each traverse gave a curve of radial distance against arbitrary units of film density and these units had to be converted to retardations from a calibration curve. This was obtained by measuring the calibration photographs, and then plotting film density against retardation. Each set of three traverses was drawn together on graph paper, and a smoothed curve was then drawn through them by hand.

* Compensation increases contrast, and enables the sign of retardation in the image to be determined.

A sample set is given in Text-fig. 2 to show the degree of smoothing that was necessary. The sixteen smoothed curves were equalized at their average peak value (3.77 Å.), and averaged to give a final experimental curve. This curve is shown in Text-figs. 3 and 4, and is an average from forty-eight traverses. The left-hand side of the curve is the outside of the ghost. The standard deviation (σ) of the sixteen smoothed curves at a retardation of 1 Å. on the left-hand side was 0.060 μ , and at 2 Å. on the right-hand side was 0.056 μ . This gives an indication of the degree of variation of the smoothed curves, and is marked on the measured curve in the figures by the two cross-bars whose total lengths are $2 \times \sigma$.



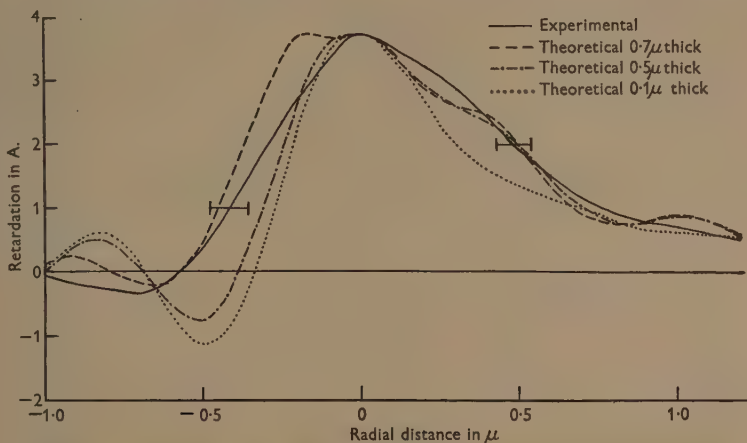
Text-fig. 2. Set of three traverses across the membrane of a ghost, and a smoothed curve.

It is not possible to work back directly from the experimental curve to the membrane thickness. The only way of finding the thickness is to calculate the theoretical curves for membranes of known thickness and then to see which of them fits best with the experimental curve. Accordingly, three theoretical curves were calculated from the out-of-focus equations (1), (2) and (6) in Appendix 1 for membranes of the same radius of the ghosts (3.0 μ), and with thicknesses of 0.1, 0.5 and 0.7 μ . The other variables in the equations were taken as follows: $\rho = 0.1 \mu$, $\lambda = 0.55 \mu$, N.A. = 1.0 and $n = 1.473$ (refractive index of glycerol). In order to compare them with the experimental curve, they were adjusted to the same peak value of 3.77 Å. by taking the following values for their coefficients of birefringence ($n_e - n_o$):

Thickness in μ	$(n_e - n_o) \times 10^{-4}$
0.1	5.90
0.5	1.47
0.7	1.29

It should be noted that changing the coefficient of birefringence alters only the height of a retardation curve, and not its shape. These three theoretical retardation curves are shown in Text-fig. 3.

A fourth theoretical curve was also computed for a very thin membrane using equations (6) and (7) in Appendix 1. With this method of working, the coefficient of birefringence required for a given peak value varies inversely with the thickness assumed for the membrane. For a thickness of 40 \AA . (which, as will be seen later, is about the thickness of the lipid layer in a ghost) the coefficient for a peak value of 3.77 \AA . is 1.43×10^{-2} . This curve has not been shown in Text-fig. 3 because it



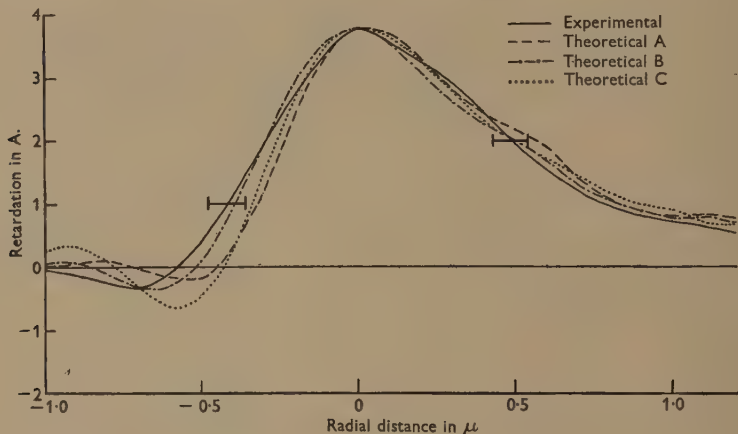
Text-fig. 3. Experimental retardation curve for ghosts in glycerol and three theoretical curves for membranes 0.1 , 0.5 and $0.7\text{ }\mu$ thick.

would be indistinguishable from the $0.1\text{ }\mu$ curve. This means that the $0.1\text{ }\mu$ curve is a limiting case and that any membrane thinner than $0.1\text{ }\mu$ would, under these conditions, give the same shape of retardation curve, though its height would of course vary with the coefficient of birefringence.

The next problem is to consider how far the experimental curve fits any of the theoretical curves in Text-fig. 3. It is too broad to be produced by a membrane of $0.1\text{ }\mu$ or thinner, and judging by its breadth it might come from a membrane between 0.5 and $0.7\text{ }\mu$. It is obvious, however, that its shape is wrong for either of these curves—the outer (left) part drops down from the peak too slowly and the first minimum comes too far out.

So far, the model membranes used to give the theoretical curves have been assumed to be uniform and to have the same birefringence throughout. In fact this assumption is very probably wrong, but in order to produce a more plausible model membrane it will be necessary to anticipate some of the later discussion and state that there is fairly good evidence of a lipid layer about 40 \AA . thick at the outer

surface of a ghost. Most of the rest of a thick ghost would be a protein gel. The retardation curve which would be produced by such a membrane can be made up by taking the curves for a 40Å. membrane (the lipid) and for a 0.5μ membrane (the protein), displacing the lipid curve 0.5μ outwards (this is equivalent to having the lipid outside the protein), and then adding the two curves together.* The two curves can be added in different proportions, so the values taken for the coefficients of birefringence determine the shape of the final curve in this case, as well as its height. Three different cases are shown in Text-fig. 4, together with the experimental curve. In each case the membrane curve is calculated for a 40Å. layer on



Text-fig. 4. Experimental curve for ghosts in glycerol and three theoretical curves for a membrane with an inner layer 0.5μ thick and an outer layer 40Å. thick.

the outside of a 0.5μ layer, but different values for the coefficients of birefringence of the layers are assumed in each case:

Curve	$n_e - n_o$ for 40Å. layer	$n_e - n_o$ for 0.5μ layer
A	0.52×10^{-2}	1.07×10^{-4}
B	0.85×10^{-2}	0.88×10^{-4}
C	1.25×10^{-2}	0.64×10^{-4}

It can be seen that curve *B* gives a satisfactory fit with the experimental curve. It is not perfect but it is well within the errors of measurement and averaging of the experimental curve. Curves *A* and *C* do not give a satisfactory fit nor do combinations of a 40Å. curve with curves thicker or thinner than 0.5μ .

These results show that the ghost membrane can be adequately represented by

* Radial orientation, producing negative birefringence, being assumed for both layers.

a model which has one layer about 0.5μ thick surrounded by a second layer about one-hundredth of the thickness of the first layer, but 100 times more birefringent. This is of course very similar to a model membrane in which the orientation and birefringence decrease on moving inwards (Ponder, 1951). There are almost certainly a number of functions of distance and orientation which would give as good a fit as curve *B*, if not better, but the choice of such a function would be so arbitrary that it is not worth doing with our very limited knowledge of membrane structure. The only definite thing that can be said about membrane models in which the orientation falls off, is that the results in this paper show that the membrane must be about 0.5μ thick and also that there must be a reasonably large degree of orientation some distance inwards, i.e. the orientation must not become vanishingly small at 0.2 or 0.3μ inwards.

Finally, it should be said that there is one other model membrane structure which gives a good fit to the experimental curve. This is a membrane composed of two 40\AA . layers of equal birefringence separated by an isotropic layer exactly 0.3μ thick. However, such a model would not give the results which are found on photographing ghosts in water, as will be described in the next section, and in any case would not agree with other experimental work on red cells.

MEASUREMENTS ON RED CELL GHOSTS IN WATER

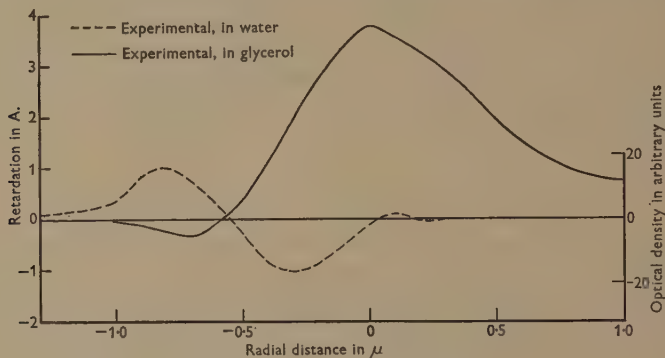
Schmitt *et al.* (1936) found that red cell ghosts in distilled water showed a weak positive birefringence (a negative cross in their terminology). It seemed desirable to confirm this and also to see what information could be gathered by measuring the breadth of the diffraction pattern at the edge of the ghosts. Since the medium has a different refractive index from the ghosts, light will be reflected from their surfaces. This light will have its plane of polarization rotated, and may possibly show a small amount of elliptical polarization. There may also be a Becke line effect from parts of the membrane which are out of focus. There is no easy way of distinguishing these effects from the retardation caused by rays travelling through the membrane, so it is inaccurate to express the light intensity of the pattern in terms of retardation and apply the full analysis used for the ghosts in glycerol. A limited amount of information, however, can still be got by measuring the breadth of the pattern.

Sphered ghosts were prepared by mixing one volume of human blood with ten volumes of distilled water, and then mounting under a cover-slip. When examined under the polarizing microscope they showed a very weak pattern at the edge. If this pattern is assumed to be due to birefringence, its inner ring was positive and narrower than the pattern in glycerol. Outside this there was a ring of the opposite sign of birefringence (as with the ghosts in glycerol), and its strength appeared to be about the same as that of the inner layer.

If the ghosts were transferred to 4% formalin or to saline (0.9% NaCl) saturated with butyl alcohol there was no change in their appearance, but in pure saline they showed a pattern which was even weaker though of the same shape. Schmitt *et al.*

(1936) found similar results with pure saline and with saline saturated with butyl and amyl alcohol.

The pattern of the edge of the ghosts in water was so weak that it proved impossible to get a photograph of them good enough for reproduction in this paper. It was, however, possible to measure the photographs on a densitometer, and four ghosts were photographed and measured in exactly the same way as the ghosts in glycerol, except that a stronger light source was used and the exposure reduced to 5 sec. The average curve from these ghosts is shown in Text-fig. 5, together with the experimental curve for the ghosts in glycerol. The ordinate of the curve for the ghosts in water is in arbitrary units of film density (very nearly proportional to light intensity) since a retardation curve would have little meaning for the reasons stated above. However, in order to give some standard of com-



Text-fig. 5. Experimental curves for ghosts in glycerol and in water.

parison with the curve for ghosts in glycerol, the peak value of film density (16) has been drawn as equivalent to a retardation of 1 Å. This is because the peak value of light intensity at the edge of a ghost in water was the same as that produced by a retardation of 1 Å. put in by a mica plate compensator.

The important fact that emerges from these measurements is that the inner lobe of the diffraction pattern at the edge of ghosts in water does not differ significantly in breadth from the outer lobe, and is considerably narrower than the equivalent part of the curve for the ghosts in glycerol. This implies that nearly all the thick inner layer of the ghost membrane which is responsible for the breadth of the main lobe of the retardation curve in glycerol must show no birefringence in water. Since it has a negative intrinsic birefringence in glycerol it must therefore have an equal and opposite positive form birefringence in water.

It is impossible to make any detailed analysis of the curve for ghosts in water for two reasons. First, the measured curve may well be broader than the true one, both because aberrations in the objective may become significant when a low refractive index mounting medium is used, and because brownian movement in

a low viscosity medium may cause the ghosts to shift during the exposure. Secondly, as stated above, it is difficult to know how much of the pattern is due to rotation and other effects rather than to birefringence.

If, however, the pattern at the edge of the ghosts is assumed to be a true positive birefringence with a maximum retardation of about 1 A., it is tempting to consider it as due to the form birefringence of a thin lipid layer at the surface. The normal Wiener formula for form birefringence cannot be applied to a single sheet, but it is possible to derive an appropriate formula using the same basic assumptions as Wiener (1912). This has been done by Dr R. O. Gandy, and the working is given in Appendix 3. The formula is calculated for a single layer of small plates arranged edge to edge, and is probably the right formula to use for a thin lipid layer with holes in it. This gives a positive form birefringence in water of about 1.1×10^{-2} if the refractive index of the layer is taken as 1.46 (the refractive index of human fat). If the layer has a negative intrinsic birefringence of 0.85×10^{-2} (p. 404), the total positive birefringence in water will be 0.25×10^{-2} . Since a layer with a birefringence of 1.43×10^{-2} would give a retardation curve with a peak value of 3.77 A. (p. 403), a layer with a birefringence of 0.25×10^{-2} would give a peak value of 0.66 A. This is of the same order as the peak value of 1 A. which is given by the ghosts in water. The objection to this argument is that the pattern for the ghosts in water is narrower than the one for a very thin membrane, so presumably the pattern has been distorted by some of the errors mentioned above.

One other deduction that can be made is that a membrane consisting of two thin lipid layers separated by 0.3μ (which would fit the experimental curve in glycerol, p. 405) would very probably not give this pattern in water. There would be both rotation and retardation at the inner layer, and it is almost certain that the resulting pattern would be broader than the one that is actually found.

THE EFFECT OF GLYCEROL

It will be argued in the discussion that the ghost membrane is probably a 2% protein gel. The argument assumes that glycerol replaces water in the intermicellar spaces of the membrane, and does not swell or shrink the structure. The grounds for this assumption must therefore be considered, especially since there have been some objections to it (Ponder, 1951).

Probably the best evidence that glycerol does not affect the membrane structure is that ghosts in glycerol have the same shape as sphered red cells (or ghosts) in saline, and very nearly the same size. The radius of ghosts in glycerol (3.0μ) is only 8% greater than that of sphered red cells (2.77μ from volume of 89 cu. μ). It is unlikely that glycerol would cause any significant change in the membrane structure without affecting shape or size.

An experiment was also done to test the effect of glycerol on the size of weak protein gels. Plasma was prepared from fresh oxalated bull's blood. The plasma was allowed to clot by neutralizing the oxalate with CaCl_2 . Two small blocks (about $2 \times 1 \times 1$ cm.) were cut out of the clot, placed in glycerol, and measured at

intervals. After 4 days in glycerol one block showed a decrease in length of 6%, and the other showed a decrease in volume of 17%. These are relatively small changes, and are in a gel which has a lower protein content than that of the ghost membrane (the fibrinogen content of cow's plasma is 0.72% (Howe, 1925), so the clot must be a gel of fibrin at about this concentration). The clot became almost transparent, showing that the glycerol had replaced the water. A similar experiment done on a 20% gelatin gel gave almost the same result—a linear shrinkage of 5% after 3 days in glycerol, and the gel turning transparent. Until, therefore, there is further evidence, it is reasonable to assume that immersion in glycerol for a few hours leaves the ghost membrane substantially unaltered.

It should be added that Schmitt *et al.* (1936) found that concentrated solutions of urea also produced a negative birefringence in ghosts, and pointed out that this indicated that glycerol was not causing birefringence by dehydration.

Glycerol is not entirely satisfactory as an immersion liquid for removing all the form birefringence, because its refractive index (R.I. = 1.47) is lower than that of protein (about 1.55). This is not, however, a serious disadvantage since form birefringence curves (birefringence plotted against R.I. of immersion medium) show that there is usually less than 10% of the form birefringence left when the R.I. of the medium is ± 0.075 from that of the micelles. Glycerol also has the advantage that its R.I. is very near that of most lipids, so that reflexion from the surface of a lipid layer will be largely eliminated. In any case, there is no other possible immersion liquid. Non-aqueous liquids are useless, and solutions of potassium mercuric iodide cause fixed ghosts to shrivel, and unfixed ones to disintegrate. Even glycerol is unsuitable with fixed ghosts as they do not become smooth spheres.

DISCUSSION

(a) *Summary of results*

Before starting the discussion it is worth recapitulating the results of the experimental work, and commenting very briefly on their validity. The main results are as follows:

(1) The birefringent membrane of ghosts in glycerol is relatively thick—of the order of 0.5μ .

(2) The outer part of this membrane is more birefringent than the inner part. A good agreement with the experimental results is given by a model membrane consisting of a layer 0.5μ thick with a negative intrinsic birefringence of 0.88×10^{-4} , covered by a layer of 40\AA . thick with a negative birefringence of 0.85×10^{-2} . Other model membranes with the birefringence stronger in the outer regions might, however, give an equally good agreement.

(3) The appearance of ghosts in water indicates that most of the membrane must have a positive form birefringence equal and opposite to the negative intrinsic birefringence. It is possible that there is a very thin outer layer which has a positive form birefringence greater than its intrinsic birefringence, so that it shows a total positive birefringence in water.

The validity of these results depend on calculations made with diffraction optics, and, as with nearly all such calculations for microscopes, a number of simplifying assumptions have to be made. These assumptions have been justified as far as possible, and are discussed in Appendix 2. It is very difficult, however, to allow for all possible errors, especially in the calculation of out-of-focus diffraction patterns, and if these errors were serious they might cause a broadening of the pattern at the edge of the ghosts, and therefore an overestimate of the membrane thickness. On the other hand, the membrane curves are comparatively insensitive to changes in the aperture of the system and to changes in shape of the out of focus patterns. Furthermore, the narrow pattern at the edge of ghosts in water indicates that errors in the optical system or in photographic technique are probably not responsible for the broad pattern in glycerol. To summarize, it is almost certain that the ghost membrane in glycerol is relatively thick, and that most of this membrane has negative intrinsic birefringence and an equal positive form birefringence. The exact thickness and strength of birefringence of the layers of this membrane, are, however, less certain.

(b) The components, and the dry thickness of the ghost membrane

It is known from chemical analysis that the two main components of washed ghosts are lipids and a protein which has usually been called 'stromatin' in the past. Boehm (1935) analysed the properties of stromatin and, although some of his results are in doubt, his general view is likely to be correct—that stromatin is fibrous in form and can, like nucleo-proteins, form gels at very low concentrations. In chemical composition, stromatin differs from other known proteins (Ballentine, 1944). Recent workers believe that there are at least two proteins in ghosts, apart from any contaminating haemoglobin (Howe, 1951; Moskowitz & Calvin, 1952). However, as Howe (1951) points out, although there may be a number of separate proteins in ghosts, it is equally true that the process of preparation involves the tearing of a tissue unit into fragments. In any case the problem is not relevant here since the argument that will be presented is equally valid whether ghost protein is a mixture or not.

Phospholipids and cholesterol make up 85% of the lipids in human ghosts (Williams, Erickson & Macy, 1941), and it is likely that a large proportion of these lipids are bound to protein (Parpart & Dziemian, 1940). Moskowitz & Calvin (1952) have separated a lipid-carbohydrate-protein complex called 'elinin' from ghosts. About 25% of elinin is alcohol-ether extractable material (presumably mainly lipids) and the rest is stromatin. Elinin is a long rod-shaped molecule with a high molecular weight. It is combined with the ether extractable lipids to form 'stromin' which is roughly equivalent to washed ghosts.

Carbohydrate is present in elinin and it has also been found before in red cells (Morgan, 1947; De Burgh *et al.* 1948). It appears, however, to be present only in small quantities.

Earlier work on the thickness (and composition) of the membranes of red cells

and ghosts has been well reviewed by Ponder (1948, 1949*a*) and it is only necessary here to give a summary in Table 1 of some of the more important results.

The amounts of dry protein in section B of Table 1, and of the 'fixed framework' in section C, are possibly underestimates since there may be a loss of matter during haemolysis and during washing (Howe, 1951). The results of Waugh & Schmitt (1940) indicate that protein and lipid can be lost, and both the anti-sphering protein and an unidentified globulin can be detected in the supernatant fluid during

Table 1. *Thickness of red cell and ghost membranes*

Reference	Thickness of layer in Å	Material	Method	Comments
<i>A. Lipids</i>				
Gorter & Grendel (1925)	c. 40	Various R.B.C.	Extraction and spreading at interface	Assuming a continuous layer
Fricke (1925)	33	Dog R.B.C.	Capacity measurements	Assuming dielectric constant = 3
Waugh & Schmitt (1940)	60	Rabbit ghosts	'Leptoscope'	Haemolysis at pH 7.4
Waugh & Schmitt (1940)	110	Rabbit ghosts	'Leptoscope'	Haemolysis at pH 6.0
Parpart & Dziemian (1940)	28	Human ghosts	Chemical analysis	From 3.94×10^{-10} mg. lipid/cell*
Parpart & Dziemian (1940)	37	Rabbit ghosts	Chemical analysis	From 3.50×10^{-10} mg. lipid/cell*
Pfeiffer (1949)	139-160	Amphibian R.B.C.	Reflected polarized light	See p. 411
<i>B. Dry protein</i>				
Waugh & Schmitt (1940)	60	Rabbit ghosts	Leptoscope	Haemolysis at pH 7.4
Waugh & Schmitt (1940)	110	Rabbit ghosts	Leptoscope	Haemolysis at pH 6.0
Ponder (1948)	37	Human ghosts	Chemical analysis	From 7.8×10^{-10} mg. stromatin/cell*
<i>C. Total dry thickness</i>				
Waugh & Schmitt (1940)	120	Rabbit ghosts	Leptoscope	Haemolysis at pH 7.4
Waugh & Schmitt (1940)	215-230	Rabbit ghosts	Leptoscope	Haemolysis at pH 6.0
Fricke, Parker & Ponder (1939)	120	Rabbit ghosts	Conductivity and weighing	From 'fixed framework' 2.2%*
Williams, Erickson & Macy (1941)	180	Human ghosts	From phospholipid content	From 'fixed framework' 3.3%*
Wolpers (1941)	150-250	Human ghosts	Electron micr.	By density
Zwickau (1941)	200-300	Human ghosts	Electron micr.	By density
Bessis & Bricka (1949)	500-1000	Mammalian ghosts	Electron micr.	By shadowing
Hillier, in Parpart & Ballentine (1952)	50-60	?	Electron micr.	?
Parpart & Ballentine (1952)	55	Rabbit ghosts	Weighing	From 'fixed framework' 1%*
<i>D. Total wet thickness</i>				
Seifriz (1927)	6000-8000	Amphibian R.B.C.	Direct observation	—

* These calculations of thickness assume: (1) a continuous surface layer; (2) density of lipids = 0.85, density of protein = 1.35, density of 'fixed framework' = 1.0; (3) surface area of rabbit R.B.C. = $110 \text{ sq. } \mu$, surface area of human R.B.C. = $140 \text{ sq. } \mu$, volume of rabbit R.B.C. = $60 \text{ cu. } \mu$, volume of human R.B.C. = $90 \text{ cu. } \mu$.

the washing of ghosts (Ponder, 1948; Moskowitz & Calvin, 1952). It is possible, however, that this loss may be compensated by contamination with haemoglobin.

It is uncertain exactly what layer was measured by Pfeiffer (1949), but his results have been tentatively placed with those on the lipids. He found the thickness of the layer by measuring the changes in ellipticity and vibration direction of polarized light reflected from the surface of amphibian red cells. The cells were stretched between micro-needles in various salt solutions. If the hypothesis about the membrane structure which is suggested later in this discussion is correct, most of the reflexion should take place at the inside and outside of the lipid layer, where there is likely to be a large change of refractive index.

The electron microscope measurements are not likely to be exact, since shadowing is difficult to interpret with a membrane which may have a curved edge, and density comparisons are never very accurate.

Bearing these comments in mind, it seems reasonable to make the following estimates of thickness for the mammalian ghost membrane from the results in Table 1:

Thickness of lipid layer	30-50 Å. (approx. 2 molecules),
Dry thickness of protein layer	50-150 Å.
Total dry thickness	100-200 Å.

These are only rough figures, and there are likely to be large differences both between different animals, and according to the method of preparation.*

(c) *The wet thickness of the ghost membrane*

The measurements in this paper have shown that the thickness of the human ghost membrane in glycerol is about 0.5μ or 5000Å . If it is assumed that glycerol replaces water in the membrane, but otherwise does not affect its structure (p. 407), then the hydrated ghost membrane is also 0.5μ thick. Although this value agrees roughly with Seifriz's figure of $0.6-0.8\mu$ for amphibian red cells, it is a good deal larger than most other estimates that have been made in the past.

A rough check on this figure was obtained by centrifuging human ghosts up to 116,000 g. (Mitchison, 1953). They were found to pack down into a layer whose thickness indicated that each ghost had a volume of $75-81\text{ cu.}\mu$. Results of the same order (human ghost volume of about $45\text{ cu.}\mu$) were also obtained in the more thorough centrifuging experiments of Ponder (1950). The volume of a sphered ghost membrane 0.5μ thick will be nearly $50\text{ cu.}\mu$. The volume of a packed ghost should therefore be of the same order as the volume found in these centrifuging experiments, if it is simply a collapsed membrane 0.5μ in thickness. If, on the other hand, the ghost were a thin walled bag with a fluid interior, it might well be

* According to Ponder (private communication), these figures are probably underestimates rather than overestimates. Many of the measurements have been made with washed ghosts, and it seems likely that the more a ghost is washed the thinner it becomes. The true thicknesses, however, are unlikely to exceed twice the value above.

expected to collapse under strong centrifuging and reach a very much smaller final volume.

Recently, Ponder (1951) has provided further confirmation of the idea that ghosts have thick walls. He has heated ghosts to about 50° C. and found that they break up into fragments whose total volume, measured in a conductivity cell, is approximately the same as that of the intact ghosts. This indicates that the ghosts are thick-walled or solid structures rather than thin hollow shells.

Since the wet thickness of the membrane is about 5000 Å., and the dry thickness is 100–200 Å., the membrane must contain at least 96% of water. Such a hydrated structure could not be produced by the lipids alone, and we must therefore conclude that it is a protein or lipoprotein gel. Taking the dry thickness of the ghost protein as 100 Å., its concentration in this gel must be about 2%. Although this is quite a low concentration for a gel, it is by no means unusual for a protein.

(d) *The structure of the ghost membrane*

In order to elucidate the structure of the ghost membrane, it is essential to establish which of the components is responsible for the birefringence. There are four possible substances: glycerol, haemoglobin, lipids and stromatin, and they will be considered in this order.

Glycerol can be dismissed at once, as there is no evidence from other work that glycerol can show birefringence. In any case Schmitt *et al.* (1936) found that urea solutions produced the same birefringence in ghosts as glycerol.

At first sight it appears that haemoglobin might be responsible, since it has a strong birefringence when crystalline, and it was certainly present in the ghosts prepared by mixing blood and glycerol. There are, however, two pieces of conclusive evidence against this possibility. First, Perutz & Mitchison (1950) have shown that when a small thickness of haemoglobin shows birefringence, it will also show anomalous colours on compensation. There was no trace of these colours in the ghosts. Secondly, there was no apparent difference between the haemoglobin-contaminated ghosts prepared by simple mixing and those prepared by the more complicated method used by Schmitt *et al.* (1936) to free them from haemoglobin. It would perhaps be desirable to check the thickness of these haemoglobin-free ghosts. They are not, however, more suitable than ghosts prepared by simple mixing because there is evidence that the process of washing out the haemoglobin also washes out some of the membrane constituents (Howe, 1951).

Having dismissed the possibility that the birefringence is due to glycerol or haemoglobin, we are left with the problem of deciding whether it is due to lipids or protein. It has been shown that the optical measurements agree satisfactorily with a model consisting of a layer 0.5μ thick with a negative birefringence of 0.88×10^{-4} surrounded by a thin layer 40 Å. thick with a negative birefringence of 0.85×10^{-2} . We have also seen that there is enough lipid in ghosts to form a layer about 40 Å. thick. This suggests that the surface layer is composed of radially oriented lipids. Such a layer would agree with electrical capacity measurements (Fricke, 1925; Cole, 1940), and there is also a good deal of evidence from other

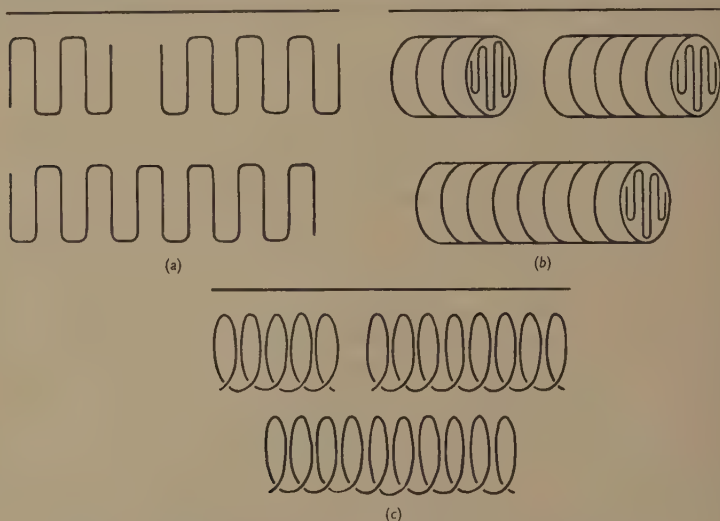
sources that the surface layer of the membrane is predominantly lipid, though with holes which would presumably contain or expose protein. First, such a structure fits many of the facts of permeability (Davson & Danielli, 1943). Parpart & Ballentine (1952) have emphasized the need for postulating water channels or pores in the membrane in order to explain the rapid penetration of water into red cells, but they point out that such pores would only account for a small part of the surface area—0.1 % in rabbit red cells. Secondly, the electrophoresis measurements of Winkler & Bungenberg de Jong (1940) show that the iso-electric point of ghosts is intermediate between that of stromatin and that of the lipids. Furchgott & Ponder (1941) conclude from I.E.P. measurements that the surface of the intact red cell is dominated by the phosphoric acid groups of kephalin and related lipids. Thirdly, the wetting properties of red cells indicate a mainly lipophilic surface (Mudd & Mudd, 1926). Fourthly, a mixed surface is indicated by the fact that it is damaged by both lipases and proteases.

Returning to the question of the birefringence, let us first consider the possibility that radially oriented lipids are responsible for all the birefringence. We can postulate a membrane structure in which the total quantity of lipids is divided into two parts. The first part is concentrated as a nearly continuous layer at the surface with a thickness of 1 molecule or about 20 Å. The second part is distributed through the 0.5 μ thick protein jelly in the form of little radially oriented micelles. At first sight, such a structure is not incompatible with the optical measurements. A 20 Å. layer would produce the same effect as a 40 Å. layer provided it had double the birefringence, i.e. 1.7×10^{-2} . If the remaining 20 Å. were spread out through 0.5 μ it would give a birefringence of 0.68×10^{-4} , which is not greatly different from 0.88×10^{-4} . There is however a serious objection to this idea. Both the lipid micelles and the monomolecular surface layer would almost certainly have to be stabilized and kept in position by a well-oriented protein framework with the chains lying at right angles to the direction of the lipid molecules (i.e. tangentially in the ghost), as in other known protein-lipid structures such as the nerve myelin sheath (Schmitt, 1950; Fernández-Morán, 1950) or retinal rods (Schmidt, 1951). Since the birefringence of oriented protein is as large, if not larger than that of lipid, and there is twice as much protein as lipid in the ghost, the presence of such a protein structure would certainly reduce the negative intrinsic birefringence drastically and very probably reverse the sign of birefringence.

If we reject the idea of scattered lipid micelles being responsible for the birefringence of the 0.5 μ layer, we must conclude that this birefringence is due to the protein. Most of the lipids can then be regarded as forming a bimolecular layer of 40 Å. at the surface of the protein layer. This is a widely accepted idea, both because of the inherent stability of such a layer and because it agrees with the electrical measurements. A birefringence of 0.85×10^{-2} is a reasonable figure for lipids. There are no figures for the birefringence of the isolated membrane lipids, but Schmitt & Bear (1937) give a value of 1.1×10^{-2} for the birefringence of myelin. It is difficult to draw any conclusions from the birefringence of 0.88×10^{-4} for the protein layer, since very little is known about the relation between birefringence

and the details of protein structure, but the degree of orientation is probably similar to that of muscle since the latter has very roughly ten times the amount of protein and ten times the intrinsic birefringence of the thick layer of the ghost membrane.

We have seen that the evidence strongly suggests that the protein layer shows positive form* and negative intrinsic birefringence, which implies radial molecular orientation and tangential micellar orientation. Now there are only two protein arrangements in a cell membrane which have been suggested in the past. One arrangement is with extended protein chains running all ways in the plane of the surface. This will not fit the facts above, unless it is assumed that the side chains are lying radially and that they determine the sign of birefringence. The evidence is



Text-fig. 6. Possible models of the protein structure in the ghost membrane.

against this, because in all extended proteins whose birefringence has been measured, the sign is determined by the direction of the long backbones of the polypeptide chains and not by the side chains. The second arrangement is that of globular proteins adsorbed on the surface. These molecules, however, have usually been regarded as statistically isotropic, like balls of string.

A new model of the protein structure is therefore necessary. One possible model would have the protein micelles running all ways in the plane of the surface, and composed of long polypeptide chains looped like a Chinese cracker or squib with their main orientation radial. A cross-section of such an arrangement is shown in Text-fig. 6a. For ease of illustration, the micelles are shown as running only in

* The positive form birefringence of ghosts is shown in a striking way in photographs of ghosts prepared for the electron microscope by Williams (1953). These are freeze-dried in a special way so that they maintain their spherical shape when dry in air. They show the strong positive form birefringence which would be expected if air replaced water as the intermicellar medium.

the plane of the paper, whereas in the membrane they would have to run all ways in the surface, in order to give statistical isotropy when viewed from above. Such an arrangement would have the required positive form birefringence due to the tangentially oriented crackers or micelles, and negative intrinsic birefringence due to the radial orientation of the polypeptide chains in the crackers.

This arrangement of looped protein chains is more than a speculation. There is a considerable amount of evidence from X-ray crystallography that proteins can form structures in which the main chains appear to lie at right angles to the length of the micelles (the 'cross- β ' pattern). Such evidence is given for egg albumin by Astbury, Dickinson & Bailey (1935); for epidermin by Rudall (1946); for keratin by Mercer (1949); and for casein, haemoglobin, zein, edestin and peanut protein by Senti, Eddy & Nutting (1943).

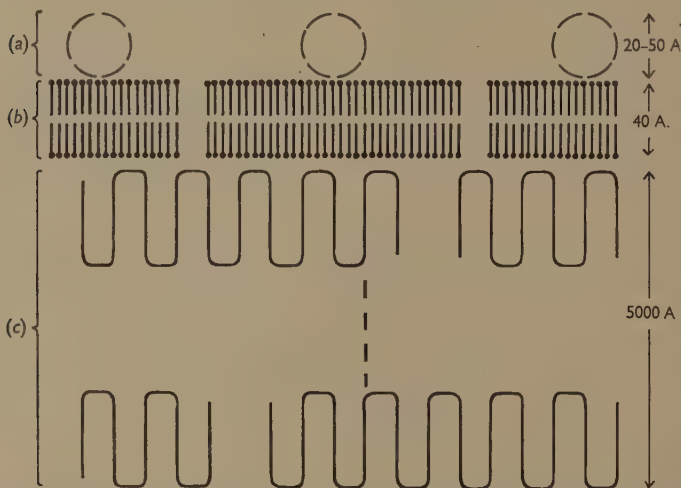
Evidence, mostly from the electron microscope, has been accumulating for some time that protein fibres may consist of a linear sequence of globular or corpuscular particles, e.g. Waugh (1944) for insulin; Farrant, Rees & Mercer (1947) for keratin; Jakus & Hall (1947) for actin. Recently, Perutz (1949), by a detailed X-ray analysis of haemoglobin, provided a possible molecular basis for this arrangement. He found the haemoglobin molecule to be a cylinder with folded polypeptide chains running parallel to the base. If these cylinders could aggregate end to end, they would give just such a linear sequence of corpuscular particles. These cylindrical columns would have the same optical properties as the looped crackers—a negative intrinsic birefringence from the looped chains and a positive form birefringence (haemoglobin crystals show a birefringence which is positive with respect to the cylinder length, but this is probably caused by the haem groups rather than by the protein). A second model of the ghost membrane can be made with these cylindrical columns instead of the looped crackers (Text-fig. 6*b*). It is a less likely arrangement than the first because the elasticity (Beams, 1947; and Norris, 1939, for amphibian R.B.C.), the gelation at low concentrations, and the properties of the extracted components (Moskowitz & Calvin, 1952), seem to indicate a more fibrous arrangement.

In view of the increasing interest in the possibilities of spirals in protein structure, it is worth pointing out that a third model can be made with the protein chains coiled in tight spirals like springs (Text-fig. 6*c*). This would also give positive form and negative intrinsic birefringence if the spirals were large enough.

The main points which have so far been made in this discussion can be summarized in a simplified model of the structure of the ghost membrane (Text-fig. 7). Most of the membrane would consist of looped bundles of protein chains forming a 2% gel about 0.5μ thick. The size of the bundles is quite uncertain. Outside this would be a bimolecular lipid layer 30–50 Å. thick, with a certain number of holes in it. Ponder (1948) has suggested that there might also be an incomplete layer of proteins outside the lipid layer, perhaps 20–50 Å. thick and probably not continuous, which would be responsible for anti-sphering and antigenic properties. The intact red cell membrane would be similar except that the protein layer would contain large numbers of haemoglobin molecules between,

and possibly within, the bundles. This is in many ways similar to the model suggested by Ponder (1948); the main difference lies in the arrangement of the protein.

This model is both speculative and simplified. The detailed structure is undoubtedly more complicated, and differs from one animal to another. This point is emphasized by Jacobs (1950) from permeability studies. The surface lipids probably form lipo-protein complexes with the surrounding proteins, which might be lying tangentially in this region. It is also uncertain whether the proteins responsible for the anti-sphering and antigenic properties form a separate layer, or are located in the holes in the lipid layer. The justification for this type of simple model is that it explains some of the facts about the general structure of the membrane, and also serves as a basis for argument and criticism.

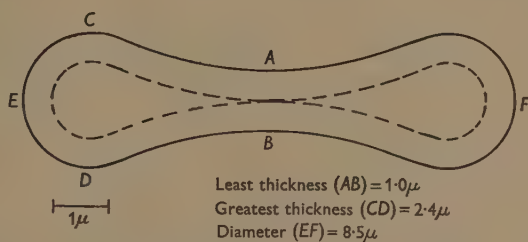


Text-fig. 7. Model of the structure of the human ghost membrane. Cross-section not to scale.
(a) Layer of antigenic and antisphering protein. (b) 'Permeability layer' of lipid. (c) 'Structural layer' of protein.

In the literature on ghosts and other cell membranes, there is a considerable difference of opinion about the use of the words 'cell membrane', 'plasmalemma', 'ultrastructure', 'cortex', etc. If the model suggested above is correct in outline, it seems reasonable to suggest the following terminology. The 'cell membrane' would include the whole membrane. The 'structural layer' would be the thick protein gel responsible for the mechanical properties of the membrane (equivalent to the 'ultrastructure' of some workers). The 'permeability layer' would be the thin perforated lipid layer (and possibly some associated protein) which is responsible for the permeability and electrical properties of the membrane.

(e) *Implication of the suggested membrane structure*

A controversy has been going on for many years as to whether the red cell is a thin-walled balloon containing haemoglobin in solution, or whether it has a solid gel-like interior. Details of the controversy are given in Ponder (1948), and only a brief summary will be given here. Examination under the microscope, whether with ordinary or dark-ground illumination, shows no internal structure and favours the balloon hypothesis. Electron microscope photographs also show the red cell as a collapsed balloon with folds in its walls (e.g. Wolpers, 1941; Bessis & Bricka, 1950). Evidence in favour of a gel-like interior comes from several sources. Red cells can be cut by microdissection into what are apparently lumps of jelly containing haemoglobin (Mason & Rockwood, 1925). Appreciable quantities of a clear jelly appear in red cells stratified by ultracentrifuging (Beams, 1947), and also in sickle cells (Ponder, 1948). If red cells are haemolysed by hypotonic media, the



Text-fig. 8. Cross-section of the human red cell with membrane 0.5μ thick.

ghosts contain more haemoglobin than is likely to remain on the surface from simple adsorption (Ponder, 1942). The concentration of haemoglobin in the ghosts is 1.3–3.8 times that in the surrounding medium, and Ponder believes it necessary to postulate an internal structure in the ghosts holding the haemoglobin. Finally, from a consideration of the shape transformations of heated human red cells, Ponder (1949*b*) suggests 'that the phenomena observed... are part of a process of disintegration of a plastic Hb-bearing "solid", rather than manifestations of the breakdown of a balloon-like structure in which the Hb is held in solution'.

This controversy can be settled if the intact red cell has a membrane of the same thickness and structure as that of the ghost. It can then be regarded as a balloon surrounded not by a thin membrane, but by one 0.5μ thick. The intact red cell would have a structure whose cross-section is shown in Text-fig. 8 (the external dimensions are those for the average human red cell taken from Ponder, 1948). The thickness at the biconcavity is 1.0μ , so the two internal edges of the membrane would be touching at this point. Within the membrane there would be an annular space with a maximum thickness of 1.4μ . The haemoglobin would have to be present not only in this space, but also throughout the weak protein gel of the membrane. This model will explain all the facts above. It would show no internal

structure under the light microscope, and would dry down to give the appearance of a thin-walled balloon under the electron microscope. On the other hand, the haemoglobin-containing jelly of the membrane would explain the evidence for a gel-like interior. It is possible that the annular space within the membrane, or the corresponding spherical space in a sphered cell, also contains a weak protein gel structure, though the absence of birefringence towards the centre of a ghost shows that such an interior gel must either lack orientation or disappear on haemolysis.

If the inner edges of the membrane touch in the biconcavity, it is much easier to understand how the red cell maintains its disc shape. It is obviously a far more stable shape with this thick membrane than it would be with a thin one.

The looped structure in the membrane may help to explain the disk \rightleftharpoons sphere transformation in red cells. During the transformation from disk to sphere the volume of the cell remains constant, but its surface diminishes by about one-third. It is not unreasonable to assume that the looped membrane structure which has been suggested for the ghost is also present in the sphered red cell. If so, there may be a tendency for the membrane to expand in area by an unlooping of the protein chains. If the surface area expands, and the volume remains constant, the sphere will start to dimple, and the dimples will deepen until their inside edges touch. This will produce a structure very similar to the normal disk shape. A further expansion might account for the long streamers in sickle cells. In a similar way, any factor which encourages the formation of the loops will cause the membrane to contract in area, and change from a disk to a sphere.

The difficulty with this scheme is that if the cell behaves like a rubber ball and the membrane keeps a constant volume, then the thickness of the membrane should diminish by one-third when the cell changes from a sphere to a disk. In that case, the inside edges of the membrane would not touch in the biconcavity. A partial solution of this difficulty comes from the observation of Waugh & Schmitt (1940) that the dried ghost shows an increased thickness of at least 15% in the region of the biconcavity* (the optical method described above is not sufficiently sensitive to detect differences of thickness of this amount). The presence of these thicker patches implies that the diagram in Text-fig. 8 is too simple, but it may explain the observation of Furchgott (1940) that in a series of disk \rightleftharpoons sphere transformations the biconcavity always reforms at the same place. It is possible, however, that this difficulty may not arise, as it may be wrong to assume that the membrane keeps a constant volume on expansion.

Since the preliminary account of this work (Mitchison, 1950) appeared three years ago, a number of comments on it have been published by other workers which ought to be discussed briefly. Ponder (1951) thinks that more weight should be placed on the results of Schmitt *et al.* (1936) on the birefringence of ghosts placed in different agents. These workers made the initial discovery of the negative birefringence of ghosts in glycerol on which this work is based. They also found a very

* This is disputed by Bessis & Bricka (1950) who find that ghosts under the electron microscope are thicker at the edge.

weak positive birefringence in saline, and a stronger positive birefringence in distilled water and in saline saturated with lipid solvents (butyl and amyl alcohol). They interpreted the increases in positive birefringence as being due to a disorientation or solution of the lipids which had been responsible for the negative birefringence in glycerol. The measurements in this paper, however, show that the thick layer which produces the birefringence in glycerol is different from the thin layer which produces the birefringence in water or the other media. It is doubtful, in any case, whether this latter birefringence is a true birefringence or a spurious one due to a rotation of the polarized light on reflexion. If we suppose that it is a true birefringence, the increase in positive birefringence on treatment with alcohol-saturated saline might be due to lipid disorientation, but it might equally well be due to a change of state of the protein, since these alcohols are also protein denaturing agents. The same effect is also obtained with formalin which is not a lipid solvent, but does affect protein structure.

Ponder (1951) gives a model of the red cell ghost which has a fairly thick lipoprotein membrane in which the concentration and orientation of lipoprotein falls off on moving inwards from the edge, and the concentration of haemoglobin increases. Such a model fits most of the data on ghosts and is perfectly consistent with the optical measurements (p. 405), subject to two provisos. First, the lipid is probably concentrated at the surface of the ghost and not spread through the membrane for the reasons given earlier (p. 413), so the material of the membrane should be protein, and not lipoprotein (except for the surface layer). Secondly, the concentration must not fall off too rapidly. The model membrane in this paper has been assumed to end abruptly 0.5μ in from the edge, but this is mainly for reasons of convenience in the mathematics, and because the choice of any particular function defining the fall off in orientation or concentration would be somewhat arbitrary. In fact, Ponder's model may well be nearer the truth.

Moskowitz & Calvin (1952) believe that what is being measured in the ghost in glycerol is an 'internal structure', probably composed of 'haemoglobin, S-protein and other materials' and not the true cell membrane. This is largely a matter of terminology, and there is no reason why the S-protein and 'other material' should not be regarded as part of the structural membrane. Haemoglobin is unlikely to be responsible for the birefringence of the thick layer for reasons given earlier, though it is presumably present in this layer. It is possible that Moskowitz & Calvin's elinin consists of most of the looped protein suggested here, both from the main part of the membrane, and from the surface layer where it would be combined with lipid to form a lipo-protein complex. These loops would almost certainly extend out on extraction of the elinin to give the long rods found with the electron microscope. It is, however, also possible that the elinin is only the extreme outer layer of the membrane, since the quantitative yield is not given; certainly elinin contains some of the outer layer because it contains surface antigens.

Finally, Parpart & Ballentine (1952) object to the idea of a thick watery membrane on the grounds that this does not fit the permeability and electrical data. This objection, however, should not apply to the model suggested above. The thin layer

at the surface would be responsible for the permeability and electrical properties of the membrane, while the thick watery layer within would not affect them.

This study of the ghost membrane has a number of implications in cell biology. It seems likely that other cell membranes may have a similar structure, and if so, the possibility of active membrane expansion which follows from a looped protein structure may be of importance in explaining the mechanism of cell cleavage and certain other processes (Mitchison, 1952). It is also possible that similar protein structures occur in cytoplasm (Swann & Mitchison, 1951).

SUMMARY

1. A new method is described for measuring the thickness of thin spherical birefringent membranes. It consists of measuring a curve of retardation against radial distance at the edge of the membrane, and comparing this curve with other curves calculated from theory for membranes of known thickness. Diffraction is taken into account.

2. This method shows that the wet thickness of the human red cell ghost in glycerol is about 0.5μ . A good agreement with the experimental results would be given by a model membrane consisting of a weakly birefringent layer 0.5μ thick surrounded by a strongly birefringent layer 40\AA . thick. It is suggested that the thick layer is a 2% protein gel, and that the thin layer is a bimolecular layer of lipids.

3. The birefringence indicates that there is radial molecular and tangential micellar orientation in the protein gel. This can be explained by an arrangement of the protein chains in looped bundles.

4. On the basis of these results a new model is put forward for the structure of the red cell membrane, and some of its implications are discussed.

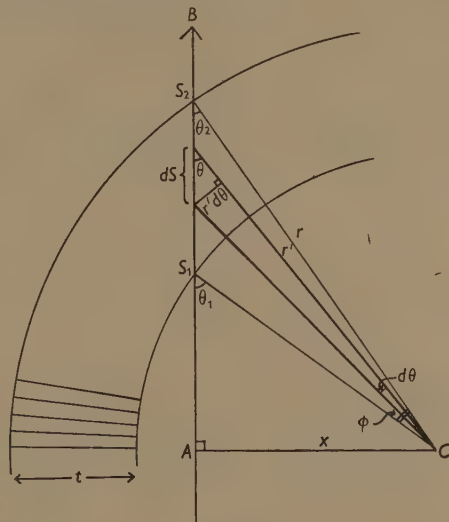
I am especially grateful to Dr R. O. Gandy, Mr A. F. Huxley and Prof. D. R. Hartree for their invaluable help with the optics and mathematics in this paper. I am also much indebted to Dr L. E. R. Picken and Prof. M. M. Swann for the many useful discussions we have had on this subject.

APPENDIX 1. FORMULAE FOR RETARDATION CURVES

(a) 'Undiffracted' retardation curves

This equation has been derived by Bear & Schmitt (1936) in the first half of a paper on the optics of the nerve myelin sheath, but it seems worth giving a simple proof of it below.

If an oriented element, with $n_e - n_o$ small, lies with its optical axis at an angle θ to a ray path, it can be shown by taking the polar equation for its velocity ellipsoid that its effective birefringence along the ray path is $(n_e - n_o) \sin^2 \theta$.



Text-fig. 9.

In Text-fig. 9 the ray AB , at a distance x from the centre C , traverses a spherical membrane of birefringent material (with a radial optical axis) from S_1 to S_2 , and has previously traversed an equal amount below. The external radius of the membrane is r and its thickness t . S_1 and S_2 subtend an angle ϕ at the centre. dS is a small element of the path, at a distance r' from the centre, and subtending $d\theta$ at the centre. If Γ'_x is the retardation along AB then:

$$\Gamma'_x = 2 \sum_{S_1}^{S_2} \sin^2 \theta dS (n_e - n_o).$$

Now

$$r' = x / \sin \theta,$$

and

$$dS = (r' d\theta) / \sin \theta.$$

Therefore

$$dS = (x d\theta) / \sin^2 \theta.$$

Therefore

$$\begin{aligned} \Gamma'_x &= 2x \int_{\theta_2}^{\theta_1} d\theta (n_e - n_o) \\ &= 2x\phi (n_e - n_o) \\ &= 2x \{ \sin^{-1}[x/(r-t)] - \sin^{-1}[x/r] \} (n_e - n_o), \end{aligned} \quad (1)$$

where $\sin^{-1}[x/(r-t)] = \frac{1}{2}\pi$ when $(r-t) < x < r$.

It should be noted that the associated ordinary and extraordinary rays are assumed to follow the same path. Bear & Schmitt (1936) conclude that for small values of $n_e - n_o$ the errors introduced by this assumption are negligible.

The first half of the right-hand side of equation (1) can be regarded as the 'effective thickness' of the membrane, corresponding to the thickness d in the normal retardation relation $\Gamma = d(n_e - n_o)$.

For spherical or cylindrical membranes where $t < 0.652r$ the maximum retardation (Γ'_{\max}) is at the inner tangent ray, i.e. $x = (r - t)$ and its value can be found by solving equation (1) with $x = (r - t)$.

For membranes where $t > 0.652r$ (up to a solid sphere or cylinder, where $t = r$), Γ'_{\max} is at the point $x = 0.652r$ and its value can be found from

$$\Gamma'_{\max} = 1.12r(n_e - n_o).$$

This is given by Frey-Wyssling (1948), who also points out that the widely quoted equation for Γ'_{\max} , given by Bear & Schmitt (1936) is incorrect.

It can be shown that

$$\int_0^r \Gamma'_x dx = \pi t(2r - t)(n_e - n_o)/4 \\ \div \pi r t(n_e - n_o)/2, \quad \text{if } t \ll r.$$

This means that the area under the retardation curve for a thin membrane is directly proportional to the radius, the thickness, and the coefficient of birefringence. This is also true for the diffracted retardation curve.

(b) 'Diffracted' retardation curves

If a spherical birefringent membrane is illuminated by a coherent beam of light in a polarizing microscope, the retardation (Γ_y) at each point on the 'diffracted' curve (retardation against radial distance) is given by the sum of the amplitudes, taking account of sign, of the contributions from neighbouring points. Each contribution will be the product of the retardation at the neighbouring point (Γ'_x) times a factor given by the amplitude of the diffraction pattern at the distance away of the point ($y - x$).

$$\Gamma_y = K \sum_x \Gamma'_x D_{y-x}. \quad (2)$$

Γ'_x can be calculated from equation (1). The diffraction function D_{y-x} is less straightforward, and three cases will be considered below. In each case the lens is assumed to be free from aberrations.

(i) General in-focus case

If all points of the membrane are assumed to be in focus the amplitude diffraction pattern will be the normal circular pattern associated with a point.

$$D_a = \frac{1}{a} J_1 \left[\frac{2\pi a (\text{N.A.})}{\lambda} \right]. \quad (3)$$

Where $a (= y - x)$ is the distance from the centre of the pattern, (N.A.) is the numerical aperture of the objective, λ is the wave-length of light, and J_1 is a first-order Bessel function.

The constant K in equation (2) is in this case $[(\text{N.A.})m^2/\lambda]$ if a square grid of points is used and m is the distance between points.

(ii) *In-focus case for a set of strips*

If the membrane can be assumed to be cylindrical, with the long axis in the object plane, then the calculations can be considerably simplified by taking the contributions from a set of thin birefringent strips, instead of from a grid of points. If all the membrane is in focus, the appropriate diffraction pattern is that for a line (Rayleigh, 1896).

$$D_a = \frac{1}{a} \sin \left[\frac{2\pi a (N.A.)}{\lambda} \right]. \quad (4)$$

In this case K is ρ/π , where ρ is the width of the strips.

(iii) *Out-of-focus case for a set of strips*

If the membrane is assumed to be composed of a set of strips, as in case (ii), but these strips cannot be assumed to be in focus, then an out-of-focus line diffraction pattern must be used for each strip, and the pattern must be that appropriate for the average out-of-focus distance between this strip and the focal plane.

Gandy (1953) has shown that the amplitude diffraction pattern of a line both in and out of focus can be given by the integral:

$$\frac{1}{2\beta} \int_{-\beta}^{\beta} \exp [ik\rho \sin(\theta - \eta)] \cos \theta \, d\theta, \quad (5)$$

where β is the semi-angle of aperture of the objective and k is the propagation constant $2\pi\lambda/\lambda$. If a is the distance between a point on the pattern and the centre of the pattern and b is the distance out of focus, then $\rho = \sqrt{(a^2 + b^2)}$ and $\tan \eta = b/a$.

In the case of a cylindrical membrane with the centre axis lying in the focal plane, each of the birefringent strips is composed of two elements lying an equal distance above and below the focal plane. The appropriate diffraction pattern is therefore given by

$$D_{a,x} = k\beta \sqrt{(R^2 + I^2)} \cos [\tan^{-1}(I/R) - kb], \quad (6)$$

where R and I are the real and imaginary parts of the integral (5) and b is the distance out of focus of the membrane for the ray at distance x from the centre (in Text-fig. 9, $b = AS_1 + S_1S_2/2$). In this case, as in case (ii), K is ρ/π . It is worth noting that for the in-focus pattern where $b=0$ equation (6) reduces to equation (4).

Unfortunately, the integral (5) is formally insoluble, and for large values of b and β numerical integration by hand takes a very long time. Prof. D. R. Hartree, however, has considerably simplified the computing (Hartree, 1953) and has prepared a programme for the automatic computer (the EDSAC) in the Mathematical Laboratory, Cambridge. This has been used to provide the numerical values used in computing the curves in this paper.

In any problem involving measurements of spherical membranes the appropriate diffraction pattern will depend primarily on the radius of the membrane and the N.A. of the objective. In the case of red cell ghosts, it is permissible to use line patterns rather than point patterns since the difference between curves calculated with equation (3) and with equation (4) is negligible. It is almost certain that there would be an equally small difference in the out-of-focus case between curves using equation (6) and those using equation (3) modified to allow for out-of-focus point patterns. No attempt, however, has been made to find these latter curves owing to the prohibitive length of the computing which would be needed.

On the other hand, it is not permissible to assume that all parts of the membrane are in focus, since substantial contributions to the retardation curve are made from parts of

the membrane well beyond the depth of focus of the objective used. All the theoretical curves were therefore computed with the diffraction patterns from equation (6). It is worth noting, however, that the broadening of the retardation curves when the out-of-focus effects are allowed for is not as great as might perhaps be expected. If these effects are ignored, and equation (4) used for the theoretical curves, the thickness of the ghost membrane comes out at about 0.7μ rather than 0.5μ .

It should be emphasized that two important assumptions have been made in deriving the equations above. First, that the objective is free from aberrations, and secondly, that the membrane is illuminated by coherent light. The validity of these assumptions is considered in Appendix 2 with regard to the particular optical system used for the ghost photographs.

(c) Retardation curves for very thin membranes

If a membrane is very thin compared to the breadth of the diffraction pattern, equation (2) will tend to give undue weight to the maximum retardation at the inner edge. Instead of using the height of a strip under the retardation curve as the multiplying factor for the diffraction pattern, it is better to use the area of the strip in the following way. This approximation is only valid for very thin membranes.

In Text-fig. 10, the 'effective thickness' $E = 2x\delta\theta$ (from equation (1)). For a thin membrane ($t \ll r$),

$$\begin{aligned}\delta\theta &= \sin \delta\theta = y/r \\ &= t \cot \theta / r.\end{aligned}$$

But

$$\cot \theta = x/\sqrt{(r^2 - x^2)}.$$

Therefore

$$\delta\theta = tx/\{r\sqrt{(r^2 - x^2)}\}.$$

Therefore

$$E = 2x^2t/\{r\sqrt{(r^2 - x^2)}\}.$$

Let the area under the undiffracted retardation curve from $x=a$ to $x=b$ be A .

Then

$$\begin{aligned}A &= (n_e - n_o) \frac{2t}{r} \int_b^a \frac{x^2}{\sqrt{(r^2 - x^2)}} dx \\ &= (n_e - n_o) \frac{2t}{r} \left[-\frac{r^2}{2} \sin^{-1} \frac{x}{r} - \frac{x}{2} \sqrt{(r^2 - x^2)} \right]_b^a.\end{aligned}$$

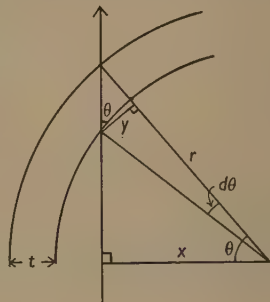
Then the diffracted retardation curve is given by

$$\Gamma_y = K \sum_x A_x D_{y-x}. \quad (7)$$

If D_{y-x} is given by equations (4) or (6), then $K = 1/\pi$.

APPENDIX 2. VALIDITY OF THE OPTICAL ASSUMPTIONS

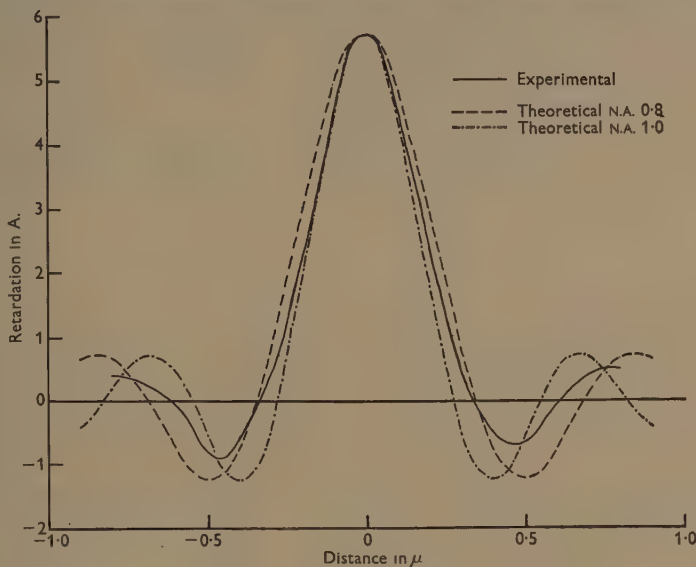
A number of optical assumptions were made in the derivation of the membrane equations and their application to the ghosts. The most satisfactory way of confirming the validity of these assumptions would have been to test the theoretical curves against a model membrane of known thickness and of the same dimensions as the ghosts. Such a procedure would have given a simultaneous check on the validity of all the assumptions,



Text-fig. 10.

but unfortunately it proved impossible to find a suitable model membrane. As a result, it was necessary to test some of the assumptions separately.

The first main problem was to see whether the in-focus line diffraction pattern defined by equation (4) in Appendix 1 was in fact the diffraction pattern given by a line under the optical system used. It was possible that this pattern might have been seriously distorted by wrong assumptions about the N.A. of the system, the presence of lens aberrations, or the use of white light rather than monochromatic light. The best method of testing this seemed to be to photograph a linear diffraction pattern and see whether it agreed with theory. The main piece of the tail of a bull sperm makes a good test object, since it acts as a birefringent line whose thickness is small compared to the diffraction pattern.*



Text-fig. 11. Experimental curve for sperm tails and two theoretical curves for N.A. of 0.8 and of 1.0.

Accordingly, dried bull sperm were photographed (Pl. 1*b*) and measured in the same way as the red cell ghosts except in two respects. First, a mixture of paraffin and methyl salicylate (R.I. = 1.50) was used instead of immersion oil (R.I. = 1.52) to allow for the fact that some of the ghosts may have been photographed through a layer of glycerol. Secondly, the plates were measured on a Hilger microphotometer with a long thin slit (0.05μ by 1μ at object), which is more suitable for a linear diffraction pattern than the densitometer used for the ghosts. The average experimental curve is shown in Text-fig. 11, together

* Randall & Friedlander (1950) from EM photographs of ram sperm give diameters of 0.50 – 0.53μ for the main piece, and 0.15 – 0.20μ for the tail end or axial filament. If a bull sperm is similar to a ram sperm, the main piece would seem to be too broad for a line source. It does, however, appear to act as a line source, because it gives a linear diffraction pattern which is the same as that produced by the thinner tail end. We must therefore presume that only a part of the main piece is acting as the major source of birefringence. This may well be the axial filament, but, even if the whole tail is birefringent, most of the birefringence would appear to come from a strip narrower than its diameter, since the tail is circular in cross-section (possibly semicircular when dried on to a surface).

with two theoretical linear diffraction patterns calculated from the $\sin x/x$ formula (equation (4), Appendix 1) with N.A.'s of 1.0 and 0.8, and λ as 0.55μ . The average curve would give the best fit to a theoretical curve calculated for N.A. 0.9, though the outer lobes are rather smaller in amplitude.

A confirmation of these results was obtained by measurements on teased asbestos fibres which, when thin enough, also yield linear diffraction patterns. The fibres were mounted in Canada Balsam (R.I. = 1.53) and Euparal (R.I. = 1.48-9), and the distance between the two first minima of the diffraction pattern was measured with a screw micrometer eyepiece (the rest of the optical system being the same as for the ghosts and the sperm). This was much quicker than measuring photographs, though a photographic check on five fibres showed that the micrometer measurements were consistently less than the photographic ones by an average of 5%. Thirty-five fibres were measured, and the results for the lowest ten in each medium are given below (the lowest ten were taken because the distance between minima cannot be smaller than in the theoretical diffraction pattern, but can become larger as the fibre thickness increases):

Medium	Av. distance between minima in μ	Standard deviation in μ	Av. distance plus 5 %	Equivalent N.A.
Balsam	0.89	0.02	0.93	0.86
Euparal	0.88	0.05	0.92	0.87

These results agree with those from the sperm where the measured curves have an equivalent N.A. of about 0.9.

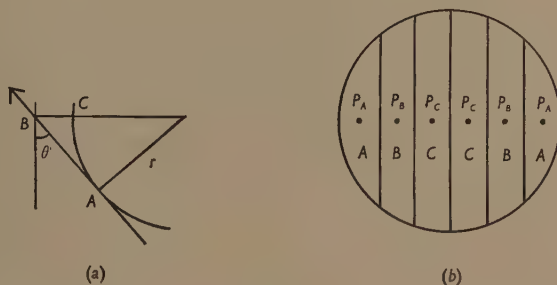
These two experiments show that in this optical system the in-focus diffraction pattern of a line does not show a large departure from what should be expected from theory, and that lens aberrations and white light do not cause serious errors. It was, in any case, unlikely that lens aberrations would have been large. The objective was stopped down from N.A. 1.3 to 1.0 which would make a considerable reduction in spherical and chromatic aberration. Coma and chromatic difference of magnification were likely to be negligible since the ghosts were always centred in the field, and only occupied a small part of it. As for the question of light, although it would have been better in theory to have used monochromatic light, in practice the ghost photographs could not have been taken in monochromatic light owing to the much longer time exposures which would have been necessary.

When calculating the theoretical membrane curves with the out-of-focus diffraction formula it seemed better to use the true N.A. of the objective (1.0) rather than the empirical value of 0.9 found above, since this broadening of the linear patterns might not have been due to a real decrease of the N.A. Even if the lower N.A. had been used, however, it would have made a negligible difference to the final theoretical curves. An earlier calculation with an N.A. of 0.625—40% lower than N.A. 1.0—gave a curve for a 0.5μ membrane only 11% broader at half peak value. The reason for this is that although the breadth of the in-focus pattern is inversely proportional to the N.A., the broadening of the pattern as it goes out of focus is less with a smaller N.A.

In deriving the equations in Appendix 1 it was assumed that the membrane was illuminated by a parallel and coherent beam of light. Since a condenser was used in the microscope this was not strictly true, and it is therefore necessary to consider how much difference this would have made.

There are two effects of using a cone of light from a condenser rather than a parallel beam. The first effect is on the summation of diffraction patterns, which with coherent

illumination should be added in amplitude, and with random phase illumination should be added in intensity. In a microscope it is usually assumed that if the condenser N.A. is small compared to the objective N.A. the illumination is coherent, while if the two N.A.'s are equal the illumination approaches random phase. With the optical system used for photographing the ghosts the condenser N.A. was $1/4$ of the objective N.A., which would probably be regarded as small. It is impossible to be strictly quantitative about this, but there are two indications that the assumption of coherence in this case is justified. First, Hopkins & Barham (1950) have developed the theory of a 'partial coherence factor'. This varies from 1 when the illumination is coherent (condenser N.A. very small) to 0 when the illumination is in random phase. They consider an area to be coherently illuminated when this factor is greater than 0.88. With the optical system used for the ghosts this factor is 0.85 for points 0.4μ apart (and increases as the separation decreases). In the case of the theoretical membrane curve for a 0.5μ thick membrane, 70% of the contributions to the peak value come from parts of the membrane which are 0.4μ apart and less. Secondly, the diffraction pattern given by the sperm tails in Text-fig. 11 resembles the theoretical pattern calculated from the sine function which assumes coherence. The theoretical pattern for a line in random phase illumination is given by a Struve function and is quite different from the sperm tail curves, e.g. the tail of such a pattern never descends below zero.



Text-fig. 12.

The second effect of using a cone of light from a condenser is not connected with diffraction and is a purely geometrical one. Beams of light coming through the membrane at different angles will produce undiffracted retardation curves which are displaced relative to each other, and it is possible that this might broaden the diffracted curves. Bear & Schmitt (1936) concluded that this effect could be ignored with a small condenser N.A., but it seemed desirable to confirm this point by an approximate calculation with ray optics.

Consider an oblique ray of light AB at an angle θ to the vertical, and touching a birefringent cylindrical membrane of radius r at the point A (Text-fig. 12a). This ray will produce the same retardation (zero in this case) as the vertical ray touching the membrane at C , but the retardation will appear to come from the point B in the focal plane. A similar argument applies to all rays parallel to AB . They will produce a retardation curve identical to that produced by vertical rays (neglecting questions of focus), but which will appear to be displaced outwards from the centre by the distance BC . It can be seen that

$$BC = r(\sec \theta - 1).$$

In the case of the ghosts, each point in the focal plane was assumed to be illuminated by a cone of light from the condenser. The apex of the cone was at the point in the focal plane, and the apex angle was the angle of aperture of the condenser. A circular cross-section of the cone was divided into six strips of equal breadth, as in Text-fig. 12*b*. The strips were assumed to be parallel to the straight edge of the cylindrical membrane. The light from each strip (from a series of cones) would produce a retardation curve displaced by an average amount equal to $r(\sec \phi - 1)$, where ϕ was the half angle subtended at the apex of the cone from the mid-point of the strip (P_a , P_b or P_c). Values of the displacement were calculated for each of the strips *A*, *B* and *C*, assuming a half angle of the condenser of 9.5° (N.A. 0.25 in oil), and r as 3μ . These were:

Strip	Displacement in μ
<i>A</i>	0.028
<i>B</i>	0.011
<i>C</i>	0.001

A series of six undiffracted retardation curves were then drawn, calculated from equation (1) for a membrane 0.5μ thick. The curves were displaced by the amounts above, and were also weighted by the areas of the strips ($A:B:C = 0.525:0.875:1$). They were then summed, and the resulting curve was 'diffracted' with the linear in-focus function (equation (4), Appendix 1). The final diffracted curve was so nearly identical with a curve calculated in the same way but assuming parallel light, that they would not be separable at the scale used for the graphs in this paper. It is reasonable to assume that the difference would also be negligibly small if the out-of-focus diffraction function was used. We can therefore conclude that the effect of correcting for an illuminating cone is small enough to be ignored, and that the illumination can be treated as parallel.

There are two main limitations in this calculation. First, it was done with ray optics where it is probably correct to assume that the cone of light should be that delivered by the condenser. It would be better if it could have been done with wave optics where the acceptance cone of the objective could also have been considered. Secondly, it neglected the fact that the contributions from the oblique rays were not only displaced, but might also have been out of focus. It can be shown, however, that the maximum distance out of focus (*AB* in Text-fig. 12*a*) for the outer strip *A* is within the depth of focus of the objective, even with the stringent quarter-wave criterion.

There is one final question—whether the out-of-focus diffraction patterns might not be distorted by lens aberrations. It is impossible to test this experimentally with a line source like the sperm tails because the formula for the out-of-focus pattern (equation (6), Appendix 1) assumes an object symmetrically placed above and below the focus. However, this effect is not likely to be serious since the in-focus pattern does not seem to be distorted by lens aberrations. Also it would need a very large distortion of the out-of-focus patterns to make any significant difference to the theoretical curves.

APPENDIX 3. FORM BIREFRINGENCE OF A SMALL THIN PLATE

By R. O. GANDY

Department of Mathematics, University College, Leicester

If an object whose dimensions are small compared with a wave-length is placed in a monochromatic electromagnetic field \mathbf{E}_0 , then the resultant field \mathbf{E}_1 may be regarded as the combination of the incident field \mathbf{E}_0 , and the 'scattered field' \mathbf{E}_2 ; and it is well known that the scattered field is that which would be produced by a dipole source of a strength d situated within the object. If the object is being examined in a polarizing microscope only the field produced by the component of this dipole which is perpendicular to the incident field will contribute to the image. By comparing the strengths of this component of the equivalent dipole for a refractive and for a birefringent object we arrive at the formula for the effective birefringence (form birefringence) of the refractive object. Since the dimensions of the object are small compared with a wave-length the calculations can be carried out using only electrostatic theory.

In Text-fig. 13 we suppose that the incident field is propagated along the z axis, and that its electric vector \mathbf{E}_0 , parallel to OP , makes an angle of 45° with the x axis. The dielectric constant of the surrounding medium is k_1 . The object considered is a parallelepiped with dimensions e, ρ, t along the x, y, z axes respectively, and we assume: $e \ll \rho \ll \lambda$ and $t \ll \lambda$, where λ is the wave-length.

We require the component of the equivalent dipole in the direction OA for two cases:

Case A. The object is isotropic, with dielectric constant k_2 .

Case B. The object is uniaxially birefringent with its optical axis parallel to the x axis; its dielectric constants are k_3, k_1, k_1 in the x, y, z directions.

The incident field may be resolved into fields of strength $E_0/\sqrt{2}$ parallel to the x and y axes, and these two fields can be dealt with separately. For the field parallel to the x axis, it can be shown that the moment d_x of the equivalent dipole is given approximately by

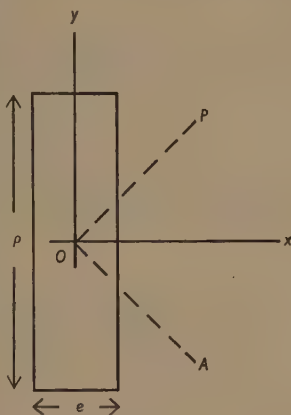
$$\text{case A} \quad d_x = \frac{E_0 V}{4\pi\sqrt{2}} \left(1 - \frac{k_1}{k_2} \right),$$

$$\text{case B} \quad d_x = \frac{E_0 V}{4\pi\sqrt{2}} \left(1 - \frac{k_1}{k_3} \right),$$

where V is the volume of the object ($= \rho \times e \times t$). For the field parallel to the y axis, it can be shown similarly that the moment is given approximately by

$$\text{case A} \quad d_y = \frac{E_0 V}{4\pi\sqrt{2}} \frac{2(k_2 - k_1)}{(k_2 + k_1)},$$

$$\text{case B} \quad d_y = 0.$$



Text-fig. 13.

When the incident field is E_0 parallel to OP then the total resultant dipole has components d_x, d_y parallel to the x, y axes. Hence the component parallel to OA is

$$d_A = d_x/\sqrt{2} - d_y/\sqrt{2}.$$

Then:

$$\text{case A} \quad d_A = -\frac{E_0 V}{8\pi} \frac{(k_2 - k_1)^2}{k_2(k_2 + k_1)},$$

$$\text{case B} \quad d_A = \frac{E_0 V}{8\pi} \frac{(k_3 - k_1)}{k_3}.$$

If the birefringent object is chosen so as to be equivalent to the refractive object then we must have

$$\frac{k_3 - k_1}{k_3} = -\frac{(k_2 - k_1)^2}{k_2(k_2 + k_1)}.$$

Hence the form birefringence of the refractive object can be found in terms of the refractive index of the medium n_1 , and of the object n_2 (provided $k_3 - k_1$ is small)

$$n_e^2 - n_o^2 = -\frac{(n_2^2 - n_1^2)^2}{(n_2^2 + n_1^2)} \cdot \frac{n_1^2}{n_2^2}.$$

This formula also applies to a number of small plates lying in the y, z plane, and so approximates to the condition of a thin lipid layer with holes in it.

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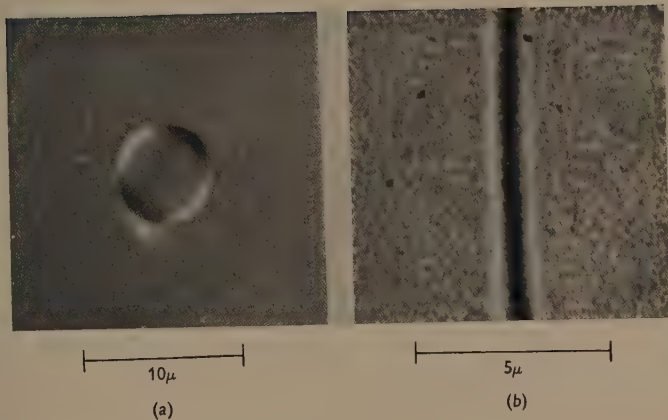
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EXPLANATION OF PLATE

- (a) Human red cell ghost in glycerol. Polarized light, with compensation. See p. 400.
- (b) Tail of dried bull spermatozoon. Polarized light, with compensation. See p. 425.



MITCHISON—A POLARIZED LIGHT ANALYSIS OF THE HUMAN RED
CELL GHOST



DISTRIBUTED 'END-PLATE POTENTIALS' OF CRUSTACEAN MUSCLE FIBRES

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(Received 29 January 1953)

Crustacean muscles are known to produce powerful contractions without propagated action potentials (Wiersma, 1941*a*; Katz & Kuffler, 1946; Katz, 1949). It has been suggested (Wiersma, 1941*a*) that this type of response is made possible by the presence of nerve endings which are widely distributed along the surface of the muscle fibres. When nerve impulses arrive at the endings, a local depolarization is produced in the muscle fibre and in turn gives rise to contraction. This depolarization will be referred to as 'end-plate potential' (e.p.p.) by analogy with the e.p.p. of vertebrate muscle. A sufficiently large e.p.p. initiates a spike potential (Katz & Kuffler, 1946), which serves to strengthen the muscle response and, probably, to level out local inequalities in the fibre rather than to conduct excitation (cf. Fatt & Katz, 1953).

If this picture of crustacean muscle response is correct, then the e.p.p. should be much more widely and uniformly distributed along individual fibres than, for instance, in frog or cat muscle where e.p.p.'s are localized at one or a few discrete junctional points of each fibre. Previous recordings of crustacean e.p.p.'s were open to criticism on various grounds: they were complicated by the pennate arrangement of the fibres within the shell and the usual difficulties of interpreting external records made in a volume conductor. To simplify conditions, Katz & Kuffler (1946) reduced the nerve supply by section of axon branches, so that only a small bundle of exposed muscle fibres remained active. Under these conditions they showed that simple, sharply localized, e.p.p.'s could be obtained. But it is possible that their reduced active bundle had also been partly denervated and deprived of adjacent nerve foci. To obtain evidence which is free from these objections, the intracellular recording technique was applied, and the e.p.p. of crustacean muscle mapped out along individual muscle fibres.

METHODS

Preparations. The muscle used most frequently was the extensor of the carpopodite of *Portunus depurator*. Dissection, mounting and application of the micro-electrode have been described in detail by Fatt & Katz (1953), where further references to the intracellular recording technique are given. In some experiments, the flexor of the dactylopodite of *Carcinus maenas* was used, its muscle fibres being locally exposed by opening the shell, and the limb being 'perfused' antidromically

with crab Ringer (see Pantin, 1934; Fatt & Katz, 1953), flowing through the tip of the dactylopodite.

Stimulation and recording. The nerve was stimulated in the saline bath by applying an external capillary electrode. A platinized platinum wire was used inside the capillary tube, and a platinum sheet was affixed to the wall of the bath to serve as an indifferent electrode. The stimuli consisted of repetitive rectangular current pulses of less than 1 msec duration.

When the extensor of the carpopodite was used, the nerve was stimulated *in situ* lying on the surface of the muscle. A recording electrode was inserted into one of the more distally situated muscle fibres, selecting successively several points along some 5–6 mm. of its length. The stimulating electrode was placed on the nerve near its proximal entry into the meropodite, well away from the muscle fibres to be examined. The frequency of stimulation was 20–30 per sec., at which little or no movement occurred. According to Wiersma (1941*b*), the fibres of the extensor muscle are supplied by two motor and one inhibitor axons. These axons must have been stimulated together, and no attempt was made to discriminate between them. The response, however, was always of all-or-none type: the e.p.p. occurring above a certain threshold and showing no increase as the stimulus was strengthened.

Each record resulted from many successive sweeps (synchronized with the frequency of stimulation), and was obtained a few seconds after the commencement of stimulation when individual e.p.p.'s had built up to a steady amplitude.

RESULTS

During preliminary experiments nerve-muscle preparations of crabs, lobsters and hermit crabs were explored by inserting a recording electrode into exposed muscle fibres and stimulating the nerve repetitively. It appeared at the outset that 'e.p.p.'s' could be seen at every point of the muscle which was impaled. The potentials built up in amplitude with repetitive stimuli, and summated at high enough frequencies as previously described (Katz & Kuffler, 1946). Typical 'e.p.p.'s' recorded with an internal electrode are shown in Figs. 1 and 2. There were large differences in amplitude in different fibres, but it was never necessary to search for a 'focus', as is the case in most vertebrate muscles, and it appeared from the ubiquitous presence of e.p.p.'s that the innervation of crustacean muscles must indeed be widely distributed along all the fibres.

Further observations were made on the extensor muscle of the carpopodite which possesses long, and easily accessible, fibres. Resting and 'end-plate' potentials were measured at several points of the fibres covering as much of their length as possible. Examples are shown in Fig. 3, where the amplitude and duration of the rising phase of the e.p.p. have been plotted. The amplitude of the e.p.p. varied to some extent along each fibre, but in only one out of fifteen fibres did the e.p.p. vanish near one end. In any one of the other fourteen fibres, the range of variation of e.p.p. amplitudes did not exceed a factor of 2, the mean range (for a given fibre) being 1.4 (S.E. ± 0.045). It should be noted that the time of rise of the e.p.p. was approximately constant at every point.

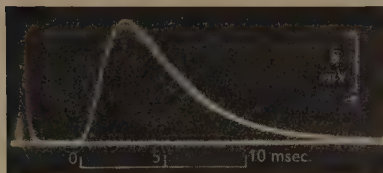


Fig. 1. 'End-plate potential' of a crustacean muscle fibre (extensor of carpopodite of *Portunus depurator*). Nerve stimulated at 25 shocks per sec. Recorded with intracellular electrode. Resting potential 66 mV.

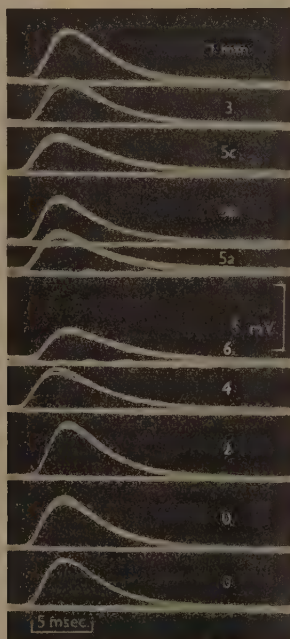


Fig. 2. Spatial distribution of 'e.p.p.'. Muscle fibre of *Portunus*, diameter 500μ . Numbers show the position of the electrode along the muscle fibre, measured in mm. from the apodeme. At 5 mm. distance, the electrode was moved transversely, being inserted at the centre (5a) and at opposite edges (5b and c) of the fibre. Nerve stimulation at 25 per sec.

In two muscle fibres, the recording electrode was also moved transversely across the fibre surface, and amplitude variations of the same order were found. Thus, in a 500μ fibre the amplitude varied, across the fibre, by a factor of 1.2, in a 285μ fibre by 1.4.

To interpret these findings, one must distinguish between two factors which cause e.p.p.'s to spread along muscle fibres: (a) electrotonic currents, and (b) distributed nerve endings. The electrotonic spread in these muscle fibres has been investigated previously (Fatt & Katz, 1953), the average length constant being

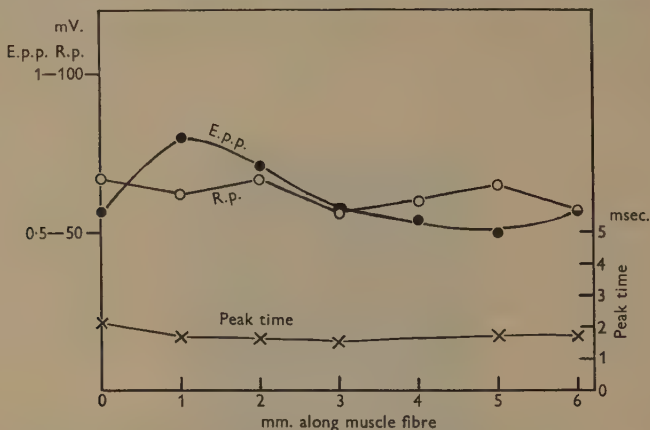


Fig. 3. Spatial distribution of 'e.p.p.' in crustacean muscle fibre. Abscissae: position of intracellular recording electrode. Ordinates: resting potential (hollow circles), e.p.p. amplitude (full circles) and time of rise of e.p.p. (crosses).

0.9 mm. (the time constant 4.6 msec.). It is clear that the much more extensive spread of the e.p.p. in these fibres cannot be attributed to their electrotonic 'cable properties' and must mainly be due to a distributed nerve supply. The constancy of the time course of the e.p.p. recorded at different points of a fibre, also suggests that electrotonic potential spread plays no important role. The results indicate, however, that there are appreciable differences in the density of innervation, or in the sensitivity of the muscle receptors, along the length and across the width of the fibre surface.

The following experiment supported these conclusions. In one muscle, after e.p.p.'s had been mapped, a block occurred in part of the axon supply (probably as a result of prolonged tetanization). When the experiment was repeated on one fibre the e.p.p. was found to have suffered a sharp 'cut-off' at a point about 2 mm. from the shell (Fig. 4). Beyond this point, the amplitude now declined sharply, and the time of rise increased, showing that the residual spread in this region was electrotonic, this part of the muscle fibre having now been deprived of a functioning

nerve supply. Incidentally, the peak size of the e.p.p. was *greater* than before, but it is not clear whether this was a 'potentiating' after-effect of the prolonged tetanus, or due to blocking of an accompanying inhibitory axon.

In several experiments, the flexor of the dactylopodite of *Carcinus maenas* was used, and the two motor axons supplying it were stimulated separately. It was found that *both* motor axons produced distributed e.p.p.'s in the same muscle fibres (e.g. Fig. 5). The e.p.p.'s due to the two axons usually differed in amplitude, and sometimes also in time course and frequency-dependence (Fig. 6), but their spatial distribution along the fibres appeared to be the same.

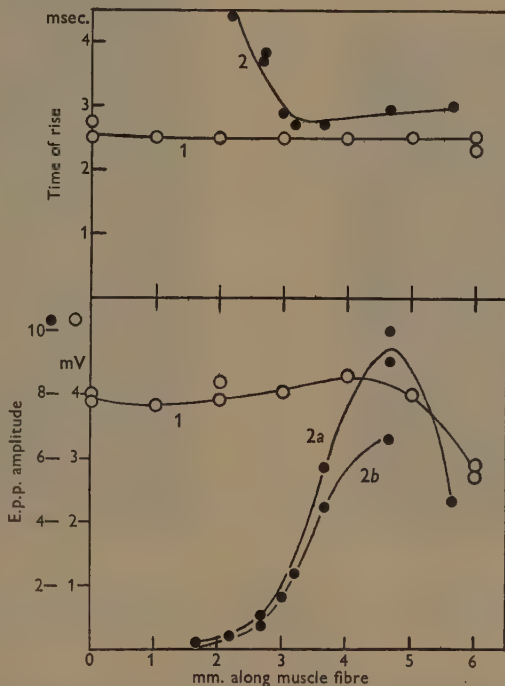


Fig. 4. Effect of partial 'denervation' of a muscle fibre. Amplitude of e.p.p. shown in lower part, time of rise in upper part. Curve 1: normal response. Curves 2: response of same muscle fibre showing effect of partial nerve block.

DISCUSSION

The present experiments support the view held by Wiersma (1941a) and van Harreveld (1939), viz. that crustacean muscles are supplied with a widespread innervation enabling them to develop powerful responses without necessarily involving propagated action potentials in the muscle fibres. As has been pointed

out (Fatt & Katz, 1953), the excitatory mechanism of the membrane (the 'spike process') which becomes operative when the depolarization exceeds some 30 mV., may serve merely to increase and equalize forces along the contractile chain, when a sudden maximum effort is required.

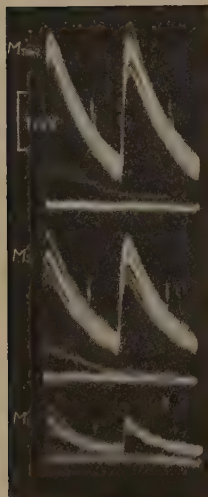


Fig. 5.

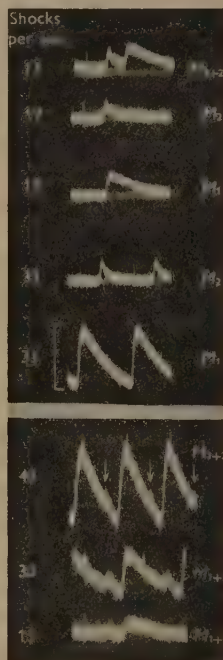


Fig. 6.

Fig. 5. Example of double motor innervation of a crustacean muscle fibre. Intracellular recording from a fibre of flexor of dactylopodite (*Carcinus maenas*). Two motor axons (M_1 and M_2) were stimulated separately or together (M_{1+2}), as indicated in the figure. The stimuli to axons M_1 and M_2 were timed so that the e.p.p.'s produced by them coincided. Frequency of stimulation 22/sec.

Fig. 6. Another example of double motor axon supply to a crustacean muscle fibre. Intracellular recording from flexor muscle of dactylopodite; stimulation of axons M_1 and M_2 as in Fig. 5. In this fibre, the responses to M_1 and M_2 differed to an unusually striking degree, both in time course and frequency dependence (M_1 response increases greatly with frequency of stimulation, M_2 does not. At the highest frequency, the relatively small M_2 potentials are indicated by arrows). Numbers show frequency of stimulation (shocks per sec.) Scale: 1 mV.

The experiment illustrated in Fig. 4 suggests that 'focal' e.p.p.'s found in 'cut-down' nerve-muscle preparations (Katz & Kuffler, 1946) may have been obtained from muscle fibres which had suffered partial denervation. In addition, however, the conditions of external recording in a crustacean limb muscle give rise to 'spatial

differentiation', and a sharpening of the observed contours of the e.p.p. (cf. the volume-conductor effect, described by Bishop (1937)). With the use of an intracellular recording electrode both difficulties have been overcome, because (a) the membrane potential of a single muscle fibre is recorded directly, and (b) there is no need to reduce the number of responding elements by micro-dissection of the nerve.

SUMMARY

1. The importance of the spatial distribution of motor nerve endings in crustacean muscle is discussed.

2. The spread of the 'end-plate potential' (e.p.p.) has been mapped out in individual crustacean muscle fibres using intracellular recording.

3. The e.p.p. is distributed over the whole length of the fibre with relatively small variations of its local amplitude. In any one of fourteen individual fibres, the size of the e.p.p. varied by less than a factor of two along 5-6 mm. of fibre length, the average range of variation being 1.4.

4. A sharp focal e.p.p. was only seen in a partially 'denervated' muscle fibre.

5. These experiments support the view that motor nerve endings are widely distributed along the length of crustacean muscle fibres.

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STUDIES ON THE MESOGLOEA OF COELENTERATES

II. PHYSICAL PROPERTIES

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(Received 5 February 1953)

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INTRODUCTION

In another paper some account is given of the histology and chemical properties of the mesogloea of certain coelenterates, among which are *Calliactis parasitica* and *Metridium senile*. It is shown there that the mesogloea of these animals contains fibres arranged in a crossed spiral pattern which is determined largely by the mechanical forces acting on the tissue (Chapman, 1953). This tissue is tough and cartilage-like to handle when it is cut out of the animal, and yet it appears to be soft and flexible in the animal during life. It would seem that the physical properties of the mesogloea might be of considerable importance in the life of the animal, yet nowhere was it possible to obtain any precise information about the isolated mesogloea; indeed, in one recent paper on the physical properties of *Metridium* body-wall (Kipp, 1940) the properties of the mesogloea are ignored. It was therefore thought to be worth while to attempt to examine the physical properties of isolated actinian mesogloea in order to see if they are consonant with the histological structure, and to see how far the physical properties ascribed by Kipp and others to the muscle may properly be ascribed to the mesogloea.

The work which is described in the present paper consists of an examination, from several aspects, of the properties of the isolated mesogloea of the two sea anemones, *Calliactis* and *Metridium*, which belong to adjacent families of the subtribe Acontiaria, so that, although they differ in their construction and habits to a certain extent, the differences are of degree rather than of kind. *Metridium* is relatively thin-walled and changes its volume enormously in an apparently spontaneous fashion, while *Calliactis* has a much thicker body-wall (*calleo*—to be thick-skinned), is exposed in life to rougher treatment and changes its volume but little. In its normal movements it has been shown (Chapman, 1949) to act as a closed fluid-muscle system.

While it was not found easy to make good ring preparations from the body-wall of *Metridium*, *Calliactis* has so thick a mesogloea that the body-wall does not corrugate much, even when the disk has been sliced off, making it possible to prepare a fairly cleanly cut ring of tissues from the unanaesthetized animal. It is possible to scrape off with a scalpel the layers inside the mesogloea, and thus to prepare a ring of mesogloea with only the ectoderm adhering; with *Metridium* it is not so easy to make a satisfactory mesogloea preparation on account of the corrugations of the body-wall and the thinness of the mesogloea. *Calliactis* was therefore

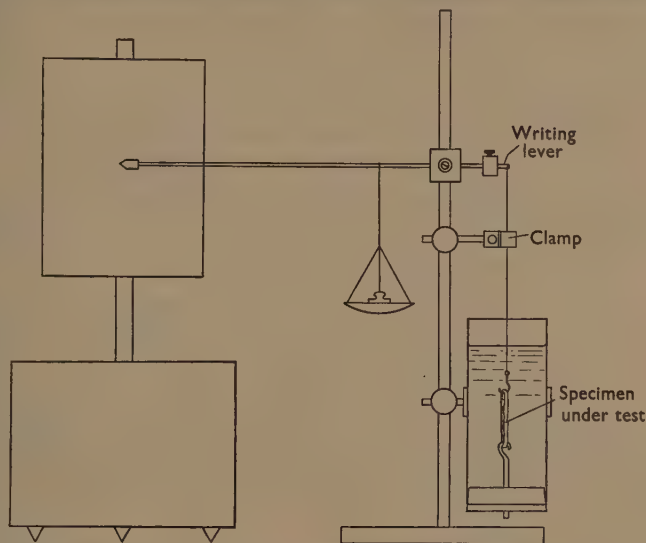


Fig. 1. Illustrating arrangement of apparatus for recording on a smoked drum the length changes of an anemone body-wall preparation.

used in the main, but the results obtained with this animal have been compared with the results of experiments made on *Metridium* in most instances. Of the viability of such body-wall preparations there is no doubt, for specimens in which the muscle layer was left intact and which had been kept in sea water at 10–15° C. for 42 hr. underwent spontaneous contractions at the end of that time.

Three main sets of experiments were carried out, the experimental details and the results for each being described separately in the following paragraphs.

LOADING EXPERIMENTS

In the first set of experiments, ring preparations of *Calliactis* and *Metridium* were kept in sea water at room temperature (usually about 12–15° C.) and were connected to a light, counterbalanced lever system writing on a smoked drum. The lever gave a fivefold increase in the length changes of the preparation. The speed

of the drum was 0.01 cm./sec. throughout the experiments. A clamp was arranged so that the length of the preparation could be kept constant without its being under tension. The experimental arrangement is shown in Fig. 1.

Experiments were made on the body-wall ring preparations of *Calliactis* cut from the middle of the column. They were prepared, as a rule, in the afternoon and were left overnight in sea water until they were used on the following day.

A series of experiments was made comparing the behaviour of preparations in which the muscle layer was left untouched, with that of preparations from which the muscle layer had been scraped with a scalpel. That there was no doubt of the complete removal of the muscle layer by scraping was shown by two methods.

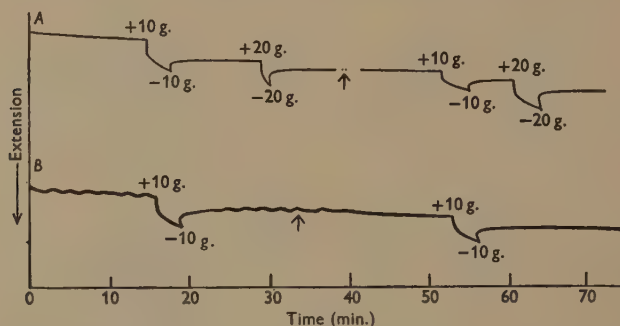


Fig. 2. (Extension) \times (Time) curves of scraped and intact pieces of *Calliactis* body-wall showing (A) absence of spontaneous contractions in the scraped, muscle-free mesogloea both in pure sea water and in Mg sea-water, and (B) spontaneous contractions in the intact body-wall test piece which are abolished by Mg sea-water. Note the similarity of the loading tests in all instances. Pure sea-water replaced by Mg sea-water at \uparrow .

Histological examination of the fixed test piece, stained in 'Azan' stain, showed no trace of the endoderm and its musculature. Secondly, a scraped test piece did not show any sign of those spontaneous contractions characteristic of intact body-wall rings which begin very soon after excision from the living animal. In addition, it was shown that the same type of loading curve was given by test pieces, both scraped and intact, immersed in pure sea-water and immersed in magnesium sea-water which completely anaesthetizes the muscle (Fig. 2). Tracings were also made with an unloaded ring of intact body-wall, prepared up to 42 hr. before use, to show the spontaneous activity of the muscle.

Comparison of the behaviour, under simple loading, of intact and scraped rings, that is, of the mesogloea plus muscle and of the mesogloea alone, showed that there were no differences between the specimens except such as could be attributed to differences in cross-sectional area of the test piece or to differences brought about by the compacting of the mesogloea which occurs on scraping. In both the scraped and intact rings on loading there was an immediate elastic extension followed by a slow elongation, rapid at first but slowing down to a nearly constant rate of

elongation. On removal of the load there was an immediate elastic shortening followed by a slow shortening, which, however, was not as great as the slow extension so that the ring did not return to its original length. Examples of simple loading tests with intact and scraped body-wall preparations are given in Fig. 3.

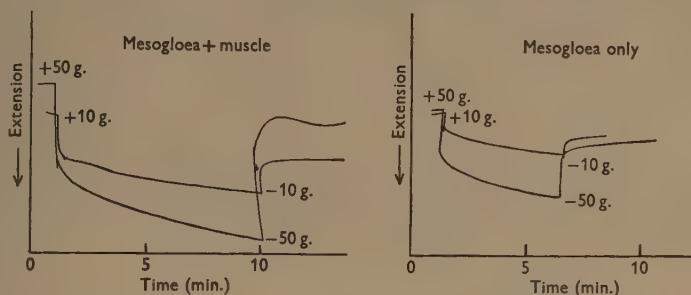


Fig. 3. (Extension) \times (Time) curves, under two different loads, of intact and scraped body-wall of *Calliactis*. Note the similarity of the viscous-elastic response of both mesogloea + muscle and mesogloea only.

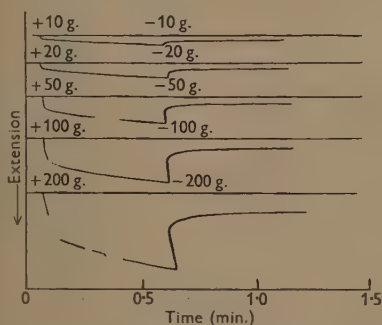


Fig. 4. (Extension) \times (Time) curves of a test piece of *Calliactis* mesogloea under different loads. The tests were performed one after the other on the same test specimen using the smallest load first.

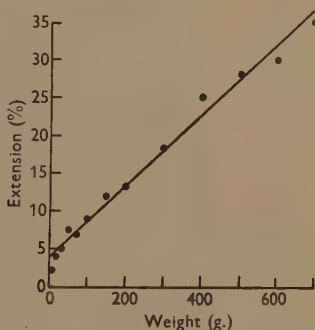


Fig. 5. Graph showing the relation between the elastic response of *Calliactis* mesogloea and the applied load.

By loading the mesogloea alone with different weights it was shown that the immediate elastic extension was nearly proportional to the load for all but small loads. It was also seen that, on unloading, the mesogloea shortened by very nearly, but not quite, the same amount as that by which it had elongated immediately on loading. These experiments are illustrated in Figs. 4 and 5, and in Table 1 in which the percentage extension of the test piece of mesogloea is shown when it is loaded with different weights.

In another type of experiment the body-wall ring was stretched by a load for 2 or 3 min. Its attachment was then clamped so that it could not shorten but it was

Table 1. *Elastic extension of Calliactis mesogloea with various loads*(Dimensions of test piece, $1.5 \times 1.0 \times 12$ mm.)

Load in g.	% immediate extension	Load in g.	% immediate extension
10	1.7	200	13.3
20	4.2	300	18.3
30	5.0	400	25.0
50	7.5	500	28.3
70	6.7	600	30.0
100	8.3	700	35.0
150	11.7	900	Breakage

The extension was measured immediately after each load had been applied. The load was removed and the new load applied. The length of time during which each load was allowed to act was insufficient for appreciable 'plastic' extension to occur. The duration of the experiment from the beginning of loading to breakage of the test piece was 13.5 min.

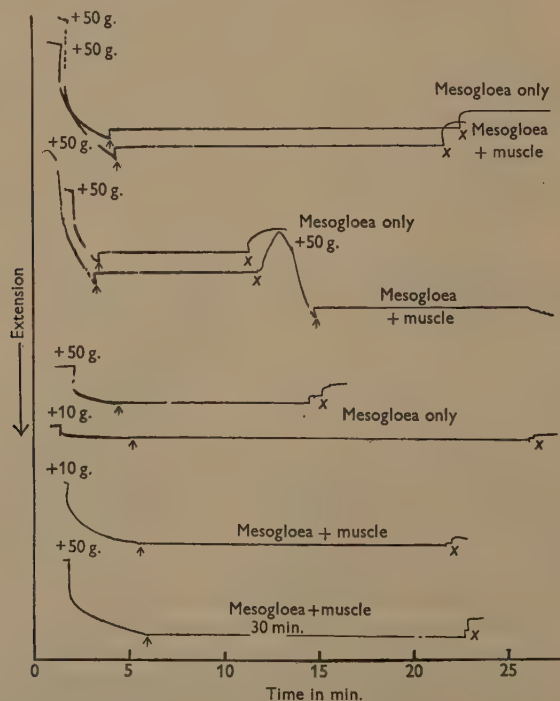


Fig. 6. (Extension) \times (Time) curves showing responses of *Calliactis mesogloea* + muscle and of mesogloea alone to maintenance at constant length after stretching. At \uparrow the specimen was clamped so that it could not extend and the load was removed. (Some backlash is shown.) At X the clamp was released and the specimen allowed to find its own length under no load. Note the similarity of response shown by mesogloea + muscle and by mesogloea alone. In both cases the specimen underwent immediate elastic shortening.

no longer subject to load. After many minutes at constant length without load (see Fig. 6) the clamp was removed and the body-wall allowed to adopt its own length without constraint. On releasing the clamp it immediately underwent an elastic shortening, a similar behaviour being shown by rings of mesogloea from which the muscle had been scraped and by intact rings.

In a third set of experiments a load was applied, stretching the tissues, the lever was returned to its position at the beginning of the experiment and then clamped. The ring was, therefore, under no tension nor was it kept at a fixed length. It was free to shorten. After a brief interval the clamp was released and the length of the preparation again registered. As will be seen from Fig. 7, both the intact body-wall

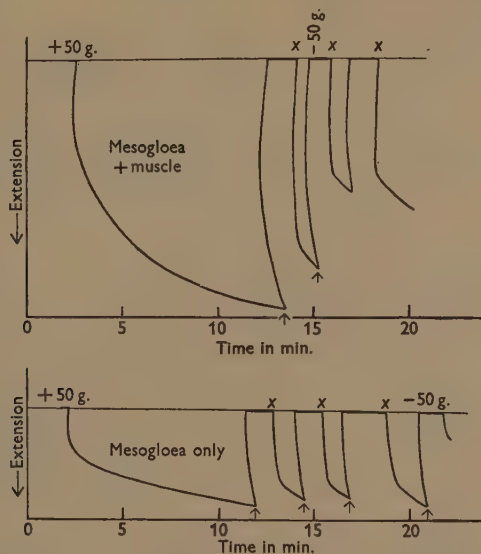


Fig. 7. (Extension) \times (Time) curve (tracing) showing responses of *Calliactis* mesogloea + muscle and of mesogloea alone to shortening under no load. At \uparrow the lever was returned to its original position at the beginning of the experiment and clamped. At X the clamp was released and the new length of the test specimen was registered under the original load.

ring and the scraped, muscle-free specimen show quite clearly that the ring recovers to a certain extent, but does not completely recover, its original length. This is, of course, only what would be expected from the simple loading tests and provides little more information about the behaviour of the preparation than they do.

Finally, spontaneous contractions of the muscle were demonstrated in specimens of body-wall prepared as long as 42 hr. before use. In another experiment spontaneous activity was shown during a loading test 4 hr. after cutting out from the animal. Such activity of the muscle may lead to confusion in the loading tests, and examples are given in Fig. 8 in which spontaneous contractions appear at the

end of a loading test. Spontaneous activity would not appear to be a significant feature of the experiments, however, because the loads employed appear to be greater than those which the muscle can exert, and because the time scale for plastic recovery of the mesogloea being much slower than that for muscular contractions the two can be distinguished.

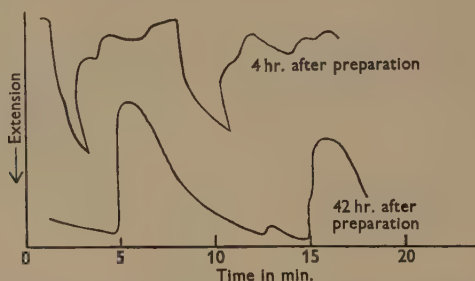


Fig. 8. Tracing showing spontaneous changes in length of a preparation of *Calliactis* mesogloea + muscle at 4 and 42 hr. after excision.

STRENGTH TESTS

Another set of experiments was made in which the mesogloea was loaded to destruction in a relatively short time, usually in about 6–7 min. These experiments were made to gain an idea of the strength of the tissue, although during the experiments the tissue was under, admittedly, artificial conditions. In addition, it was hoped that some information might be gained about the strength of the material in various directions with respect to the crossed fibrillar orientation of its fibres. Pieces of tissue were prepared as for the kymograph recordings, and their width and thickness were measured with a micrometer. Their length was measured when they were set up in a simple form of extensimeter which is illustrated in Fig. 9. An upper fixed and a lower movable clamp held the test piece tightly between pieces of moistened filter-paper. The clamps had to be screwed up tightly, otherwise the material slipped, and although the test pieces were crushed by this means they did not appear to lose their physical strength as they rarely broke at the clamps. Usually they fractured in the middle. This method of holding the specimen was therefore considered to be satisfactory because, although it caused deformity, it did not produce weakness. It should be mentioned that the dimensions of the specimen are not those which it possessed in the living animal because the fibres became compacted during preparation. The tests occupied various times, but a typical experiment took 6–7 min. from the beginning of loading to the rupture of the test piece. Strips of body-wall were cut in a longitudinal, diagonal and horizontal manner in order to test whether greater extension was possible along the length of the geodetic fibres or diagonal to them and whether those fibres were stronger along their length than they were at their interfibre junctions. The results of a number of experiments are given in Table 2.

From the table of results it will be seen that there is not a very great difference in the breaking stresses of strips of tissue cut in a horizontal, diagonal and longitudinal manner, though in *Calliactis* the extensibility of the diagonal strips is perhaps greater than that of the others.

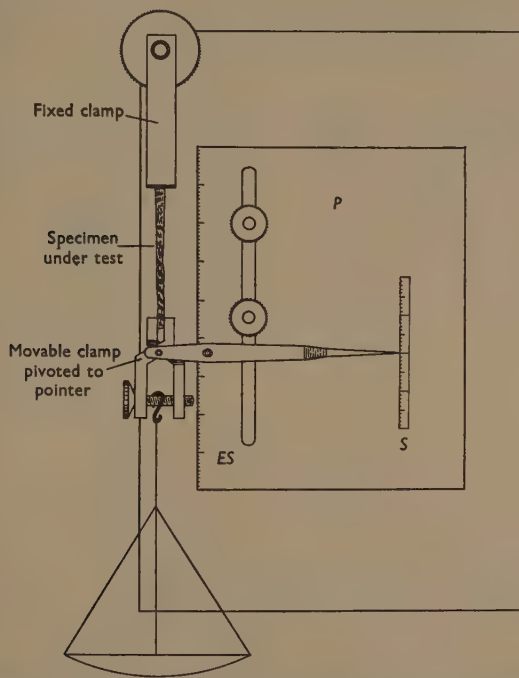


Fig. 9. Extensimeter used for strength and thermoelasticity tests of *Calliactis* and *Metridium* mesogloea. Small changes in length can be read on scale *S*, larger ones on scale *ES*. The position of plate, *P*, carrying the pointer, is adjustable to accommodate specimens of different lengths.

If all the fibres were in a 45° crossed fibrillar arrangement then diagonal strips should be relatively inextensible and vertical and horizontal strips relatively extensible. That this is not so is additional evidence that the fibres are arranged, not simply in a manner parallel to the surface, but in such a way that they can be pulled straight in a diagonal strip of tissue. This is in agreement with the arrangement of the fibres as loose sheets of warp and weft with fibrous connexions between the sheets.

The similarity of stress at the breaking point shown by strips cut in the three directions may perhaps be accounted for by the fact that when any fibrous material is stretched the fibres become orientated along the line of stretch and approach more closely to parallel orientation the more the material is stretched so that at the

end of the stretching test the test piece, no matter from which orientation it originally came, consists of fibres of nearly parallel orientation. Histological examination of stretched mesogloea supports the view that the fibres become more nearly parallel in their orientation.

Table 2. *Stress/strain tests on mesogloea of Calliactis and Metridium*

Longitudinal		Horizontal		Diagonal	
Stress at breaking point (g./sq.mm.)	Strain at breaking point (% extension)	Stress at breaking point (g./sq.mm.)	Strain at breaking point (% extension)	Stress at breaking point (g./sq.mm.)	Strain at breaking point (% extension)
<i>Calliactis</i>					
295	51	155	29	132	71
433	34	163	22	132	75
331	44	325	42	446	56
295	44	355	52	396	39
—	—	331	42	248	72
—	—	384	70	214	44
—	—	—	—	202	60
—	—	—	—	278	58
—	—	—	—	278	58
—	—	—	—	331	39
—	—	—	—	290	41
Mean					
338	43	285	43	268	56
<i>Metridium</i>					
Mean					
298	32	448	28	437	27

Similar experiments were performed on *Metridium*, the mean results of which are also given in Table 2. Despite its appearance in life it will be noted that the tensile strength of material from this animal is somewhat greater than that from *Calliactis* and that the diagonal strips are stronger and less extensible than the longitudinal ones, and of about the same strength and extensibility as the horizontal ones. This may perhaps be accounted for by the fact that, in the expanded animal, the mesogloea of *Metridium* is thinner than that of *Calliactis* so that the fibres are arranged more nearly as a two- than as a three-dimensional lattice, although the fact that the diagonal strips do show *some* extensibility points to the existence of a three-dimensional lattice in the moderately extended animal. This can indeed be seen in section and resembles the structure seen in *Calliactis* (Chapman, 1953).

THERMO-ELASTICITY TESTS

On being heated in water collagen contracts in length and eventually, at higher temperatures, loses its strength and becomes gelatinous. It was considered that if similar behaviour could be demonstrated for *Calliactis* mesogloea it would provide some additional evidence for the collagenous nature of the connective tissue. An attempt to investigate the thermo-elastic properties of *Calliactis* mesogloea was therefore made with specimens prepared in the same way as those which were used for strength tests. The test strips were placed in the extensimeter previously

described and were extended only by the weight of the extensimeter clamp (about 10 g.). The whole apparatus was immersed in a large beaker of tap water or of sea-water, and the change in length of the specimen observed at different temperatures. The length at room temperature was noted as the initial length, the temperature of the bath quickly raised to a new value and the length noted after 2, 5 and 10 min. During the 10 min. at which measurements at each temperature were made the temperature of the bath was kept approximately constant.

At 60 and 70° C., in both tap and sea-water, there is increase in length of up to 12% of the original length, but at 80 and 90° C. contraction occurs, the maximum amount being 25% at the higher temperature. This behaviour is not quite the same as that of collagen from rat tail tendons whose contraction may begin at 55–60° C. in distilled water (Partridge, 1948). There is, nevertheless, a general likeness in the response of the two materials which points to their similarities rather than to their differences. They are dissimilar in the degree of orientation of their fibres, the rat tail tendon consisting of very nearly parallel fibres, so that it might well be expected that contraction, along the length of the fibre, would be more readily observable in the tendon than in the mesogloea in which the fibres are not predominantly parallel to the length of the test piece.

DISCUSSION

There appears to be no published work on the physical properties of the coelenterate mesogloea apart from references to it such as a 'stiff jelly' or 'cartilage-like' in consistency. Its properties are easier to investigate in actinians than in other coelenterates because it is tougher and is present in sufficient quantity to make possible its isolation for direct experiment. The consistency of an intact *Calliactis* can vary from soft when open, to hard and springy when closed. The anaesthetized animal (18 hr. menthol + 3 hr. $MgCl_2$ and menthol) can be inflated by a pressure head of a few cm. of water, but when strips of the body-wall are cut out they contract and cannot easily be extended. They contract both along the long axis and at right angles to it, for, although there are only circular muscles in the column wall, the longitudinal parietals lie very close to it. A marked area on the animal measuring 2.0×1.5 cm. became 1.2×1.1 cm. on excision and 1.0×0.8 cm. on further squashing with the handle of a scalpel, a total reduction of area of 75%. And after this treatment the mesogloea was of an almost cartilaginous consistency. It is difficult to avoid supposing that the fibres have become compacted together by the loss of interfibrillar fluid, and that they cannot by themselves regain their original separation. Indeed, sections of the excised and squashed mesogloea show the fibres as a much more compact mass than in the animal fixed in the expanded condition, although in animals closed by their own efforts there is no great increase in fibre density above that of the open specimen. These observations are made under highly artificial conditions for it cannot be imagined that the mesogloea is subject to very great stretching or compacting stresses during life. Nevertheless, an examination of the physical properties as such may throw some light on the part which the mesogloea plays in the life of the animal and *vis-à-vis* the muscle with which it is so

intimately connected. In the latest and most detailed study of the physical properties of *Metridium* 'muscle' (Kipp, 1940) no account has been taken of the properties of the mesogloea, although in a ring of body-wall of *Metridium*, there is, bulk for bulk, very little muscle compared with mesogloea. The first part of Kipp's paper describes the responses of a ring of *Metridium* body-wall to loading without electrical stimulation of the muscle. All of the effects recorded he ascribes to the muscle, but it has been shown here, by experiments similar to Kipp's, that the viscous-elastic properties of the body-wall of *Calliactis* are all clearly shown by the

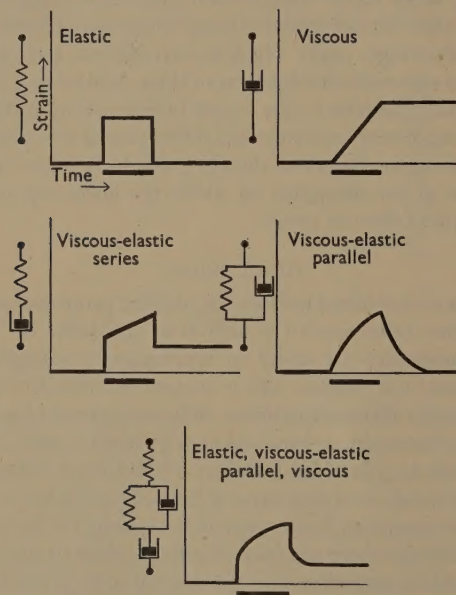


Fig. 10. Diagrams to illustrate the type of Extension (strain) \times Time curves given by different spring and dashpot models. The duration of the application of the load is marked by a heavy line beneath the abscissa.

mesogloea alone without the muscle at all. The behaviour of the tissue can be exactly described in terms of the familiar spring and dashpot combinations used to describe the mechanical behaviour of substances which show viscosity and elasticity together. In Fig. 10 is set out pictorially a number of possible combinations of springs and dashpots, together with their corresponding (extension) \times (time) curves. It can be seen that the behaviour of the mesogloea agrees with the 'elastic, viscous-elastic parallel, viscous' combination.

The description of the behaviour of the mesogloea as 'plastic' (implying a definite 'yield-point') is probably best avoided since the tissue appears to be

deformed under very small loads. While it is clear that this analogy throws some light on the structure of the mesogloea it throws none at all on that of the muscle. The analogy of the spring and dashpot is not applicable to the material of the fibres themselves, but rather to an interlacing system of fibres in a matrix.

It can be said, then, that the mesogloea can be deformed by small forces acting for a long time, that it shows a component of elasticity and that it has an element of viscosity or 'plasticity' and it can also be said that, if the unstimulated muscle has properties which are different from these they are so weak in comparison that they cannot be detected by ordinary mechanical tests but that they are, in all probability, very similar.

The tension which can be exerted by actual muscle fibre material of *Metridium* has been found by Batham & Pantin (1950) to reach 40 kg./sq.cm., and it has been shown here that the breaking strain of mesogloea material of *Calliactis* is of the same order. This would seem to imply that, in life, since the muscle fibres are present only as a single layer on the surface of the mesogloea, and since this latter is of much greater cross-sectional area than the muscle, the strength of the body-wall resides in the mesogloea rather than in the muscle.

SUMMARY

1. The physical properties of the isolated mesogloea of *Calliactis* and *Metridium* are described and the behaviour of the tissue on loading recorded. It can be compared with a spring and dashpot model.

2. It is shown that the viscous-elastic properties of the body-wall, which have previously been ascribed to the muscles, are the attributes of the mesogloea.

3. On being heated in water under a small load, isolated mesogloea of *Calliactis* contracts, at temperatures which are only a little higher than those at which vertebrate collagen contracts. This is regarded as additional evidence for the collagenous nature of the mesogloea protein.

4. It is shown that the physical behaviour of the material is consonant with the crossed fibrillar collagenous nature of the mesogloea described elsewhere.

It is a pleasure to acknowledge help from my colleagues at Queen Mary College, from the Director of the Marine Biological Laboratory, Plymouth, and especially from Dr C. F. A. Pantin, F.R.S.

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